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Analytical separations using polymetric surfactants

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ANALYTICAL SEPARATIONS USING POLYMERIC SURFACTANTS

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DEDICATION

To my mom:

Winny Wamaitha Kamande

Mom, thank you for your love, constant prayers, encouragement, guidance that gave me strength and carried me throughout this challenging journey. Thank you for instilling and exemplifying Godly principles, diligence, and perseverance without which I would have never made it.

To my dad:

Peter Ng'ang'a Kamande

Dad I want to thank you for investing in me, your confidence in me, and prayers for me that have brought me this far.

To my siblings:

David Kamande, Esther Gathoni, and Joyce Wanjiru

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Isaiah 40:31

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LIST OF ABBREVIATIONS

Abbreviation	Name
BGE	background electrolyte
BNA	1,1'-binaphthyl-2,2'-diamine
BNP	1,1'-binaphthyl-2,2'-dihydrogenphosphate
BOH	1,1'-bi-2-naphthol
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CITP	capillary isotachophoresis
CMC	critical micellar concentration
CZE	capillary zone electrophoresis
DS	dextran sulfate
EFGF	electric field gradient focusing
ESI	electrospray ionization
EOF	electroosmotic flow
GC	gas chromatography
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
H_3PO_4	phosphoric acid
MAGF	micellar affinity gradient focusing
MEKC	micellar electrokinetic chromatography

MS	mass spectrometry/mass spectrometer
$Na_2B_4O_7$	sodium borate
NaCl	sodium chloride
Na_2HPO_4	sodium phosphate
NaOH	sodium hydroxide
OT-CEC	open-tubular capillary electrochromatography
OT-CEC/MS	open-tubular capillary electrochromatography/mass spectrometry
PC-CEC	packed column capillary electrochromatography
PB	polybrene
PDADMAC	poly (diallyldimethylammonium chloride)
PEI	polyethyleneimine
PEM	polyelectrolyte multilayer
p*I*	isoelectric point
poly-L-lysine	poly(L-lysine hydrobromide)
poly-L-SUG	poly (sodium *N*-undecenyl-L-glycinate)
poly-L-SULA	poly (sodium *N*-undecenyl-L-leucine alanate)
poly-L-SUAL	poly (sodium *N*-undecenyl-L-alanine leucinate)
poly-L-SULV	poly (sodium *N*-undecenyl-L-leucylvalinate)
poly-SUS	poly (sodium undecenyl sulfate)
PSS	poly (styrene sulfonate)
RSD	relative standard deviation
SDS	sodium dodecyl sulfate
SMIL	successive multiple ionic-polymer layer

TGF	temperature gradient focusing
THF	tetrahydrofuran
Tris	tris(hydroxymethyl)aminomethane

ABSTRACT

The goal of the research presented in this dissertation is to develop effective analytical methods for the separation of both achiral and chiral analytes using polymeric surfactants. Three analytical techniques are employed to accomplish this objective: open-tubular capillary electrochromatography (OT-CEC), open-tubular capillary electrochromatography coupled to mass spectrometry (OT-CEC/MS), and micellar affinity gradient focusing (MAGF). This research work presents novel application of polymeric surfactants that contributes to improved separations of difficult to separate analytes.

The first part focuses on the application of an achiral polyelectrolyte multilayer (PEM) coating using OT-CEC. The PEM coating consisting of the cationic polymer poly (diallyldimethylammonium chloride), PDADMAC, and the anionic polymeric surfactant poly (sodium undecenyl sulfate), poly-SUS, is constructed on the surface of the silica capillary wall. The performance of the PEM coating is evaluated by use of electrochromatographic experiments and shows good selectivity for both phenols and benzodiazepines. Reproducibility of the PEM coating is evaluated by computing the relative standard deviation (RSD) of the electroosmotic flow.

The second part focuses on the use of a chiral PEM for OT-CEC separations. In this study, the cationic polymer consists of poly-L-lysine hydrobromide, while the anionic polymeric surfactant consists of poly (sodium N-undecenyl-L-leucine alanate). Optimal separation conditions of various chiral analytes are achieved by varying temperature and voltage as well as the number of bilayers and salt concentration used to construct the PEM coating.

In the third part, the coupling of an achiral PEM coated capillary to mass spectrometry using OT-CEC/MS is investigated. The PEM coating, which consists of a single bilayer of PDADMAC and poly-SUS, is used for the separation of β-blocker and benzodiazepine analytes. Optimal separation parameters are achieved by varying the background electrolyte pH and applied voltage.

Finally, the application of poly-SUS for the simultaneous concentration and separation of coumarin dyes using MAGF is investigated. The separation and focusing of coumarin dyes is enhanced by the addition of poly-SUS into the running buffer that creates a retention gradient. The effect of varying focusing times and input concentrations on peak intensity is examined.

CHAPTER 1

INTRODUCTION

Part 1 Introduction to Capillary Electrophoresis Techniques

1.1 Capillary Electrophoresis

Electrophoresis is a separation technique introduced by Tiselius in 1930 when he described the separation of blood plasma proteins in his thesis [1]. Thereafter, many advances in the use of different formats of electrophoresis were achieved after 1948 when Tiselius was awarded a Nobel Prize for his work [2]. In 1981, Jorgenson and Lukacs [3] succeeded in obtaining high resolution separations by use of a narrow fused-silica capillary for electrophoresis, after previous attempts using glass [4] and teflon [5, 6] materials failed to provide high peak efficiency separations. To this date, extensive developments in the use of capillary tubes for analytical separations in capillary electrophoresis (CE) continue to emerge.

CE is a versatile technique and has been successfully applied for the separation of analytes such as small inorganic ions, charged or neutral molecules, and large biomolecules. A variety of applications of CE have been used in analytical chemistry, forensic science, clinical chemistry, pharmaceutical research, and environmental sciences [2]. It is possible to separate a wide range of analytes using CE by simply changing the mobile phase composition. It is for this reason that CE is advantageous over other separation techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), thin layer chromatography, and slab gel electrophoresis.

There are six main separation modes used in CE and they include: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic

chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachophoresis (CITP). For the purpose of this dissertation, CZE, MEKC, CEC, and CIEF will be discussed in detail as they encompass the techniques used for this research. CZE forms the basis of the different modes of CE and, hence, most of the theoretical aspects and equations in CE are derived and based on CZE.

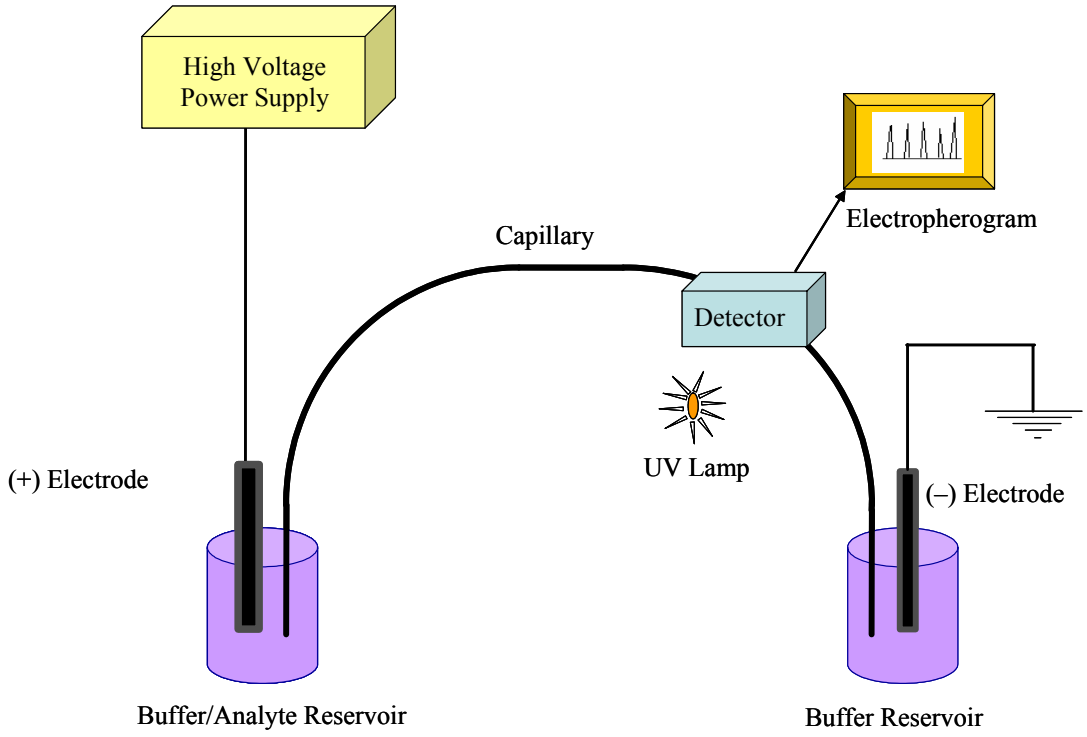

Figure 1.1 Schematic diagram of CE instrumentation.

1.2 Capillary Zone Electrophoresis

This is the simplest form of CE where the separation of charged analytes occurs in free solution with no additives under the influence of an externally applied electric field. Figure 1.1 represents a schematic diagram of the instrumentation used in a typical CE system. It consists of a fused-silica capillary, buffer/analyte reservoirs, a UV lamp, a photodiode array

detector, two electrodes that are connected to a high voltage power supply and a data output device such as computer.

An electrolyte buffer, referred to as the background electrolyte (BGE), is used to condition the capillary as well as maintain a suitable pH for the separation of analytes. The analyte is injected into one end of the capillary by replacing the buffer reservoir with the analyte reservoir. Thereafter, the analyte reservoir is replaced with the buffer reservoir and a high voltage is immediately applied across the capillary. The high voltage is used to drive the analytes from one end of the capillary to the other end via the process of electrophoresis. All analytes travel through the electrolyte buffer in discrete zones or bands and are separated based on the differences in their electrophoretic mobility. A detection window, prepared by burning the external polyimide coating on the fused-silica capillary, is used for analyte detection. The detector response yields an intensity versus time plot, referred to as an electropherogram.

1.2.1 Theory of Capillary Zone Electrophoresis

Electrophoresis may be defined as the separation of a charged solute based on its movement in the presence of an applied electric field [7]. In the presence of an electric field, E, an ion with a charge, q, experiences a magnitude of force, F_E, as shown in the equation 1.1.

$$F_E = qE \tag{1.1}$$

The ion possesses an electrophoretic velocity, v_{ep}, which is proportional to its electrophoretic mobility, μ_{ep}, and the applied electric field as shown in equation 1.2,

$$v_{ep} = \mu_{ep} E \tag{1.2}$$

As an ion moves across the electric field, it experiences an opposing force, the retarding frictional force, F_F, which is proportional the velocity, v_{ep}, of the ion and the friction coefficient, f.

$$F_F = fv_{ep} \tag{1.3}$$

During electrophoresis the ion a reaches a steady state velocity where the accelerating force is of equal magnitude to the frictional force as shown in the equation 1.4.

$$qE = fv_{ep} \tag{1.4}$$

By combining equation 1.2 and 1.4, it can be shown that the electrophoretic velocity, v_{ep}, is proportional to the charge of the ion and inversely proportional to the frictional coefficient such that,

$$v_{ep} = \frac{q}{f}E = \mu_{ep}E \tag{1.5}$$

For a spherical ion with a hydrodynamic radius, r, in a medium with a viscosity, η, the frictional coefficient, f, is given by the following expression,

$$f = 6\pi\eta r \tag{1.6}$$

Thus, by substituting equation 1.6 into equation 1.5, it can be shown that both the hydrodynamic radius and the viscosity of the medium are inversely related to the ion's electrophoretic mobility. Hence, ions with larger hydrodynamic radii migrate slower than those with smaller radii because the frictional coefficient is greater for the former. In addition, the electrophoretic mobility increases with a decrease in viscosity of the BGE.

The electroosmotic flow (EOF) is the primary force causing analytes to migrate across the capillary past the point of detection. The inner surface of a fused-silica capillary consists of silanol groups (Si-OH) that are ionized to silanoate (SiO^-) groups at pH values greater than

2 [8]. In solution, the negatively charged surface of the capillary is counterbalanced by positive ions from the buffer, forming an electrical double layer. This layer of cations is referred to as the Stern layer. Next to the Stern layer is a diffuse layer that consists of loosely held cations and anions. Figure 1.2 illustrates the types of ionic layers inside a fused-silica capillary.

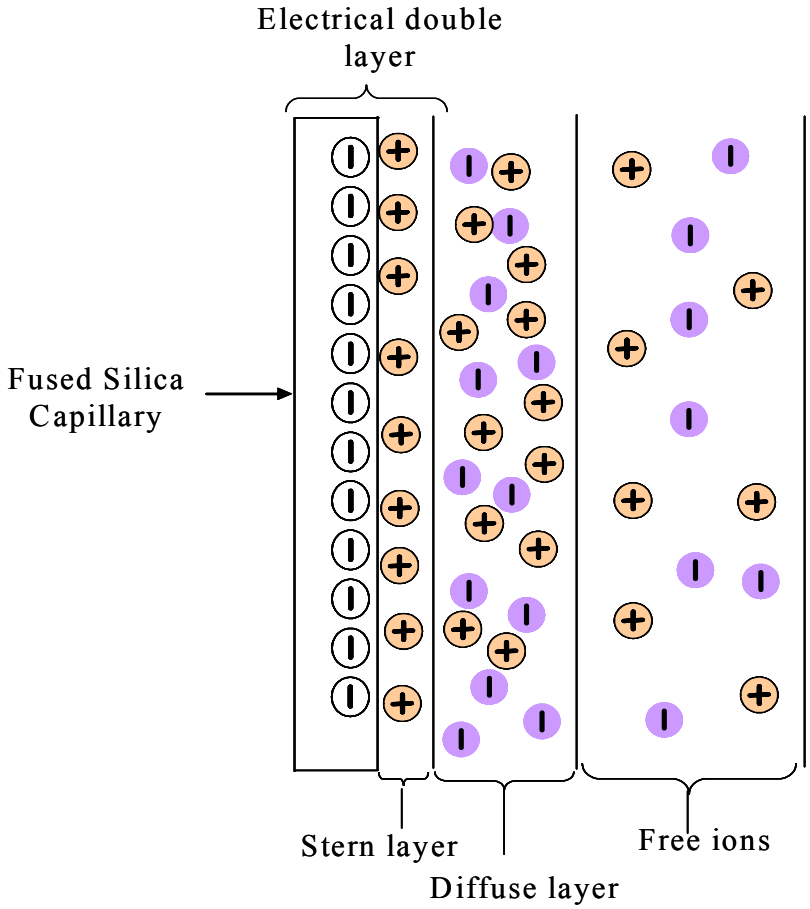

Figure 1.2 Double layer formation on the capillary wall.

On application of an electric field, the positive ions in the diffuse layer of the double layer migrate toward the cathode. In the process they drag along waters of hydration, resulting in the EOF the bulk flow of ions.

The magnitude of the EOF, v_{EOF}, can be expressed in terms of velocity by equation 1.7,

$$v_{EOF} = \mu_{EOF} E \tag{1.7}$$

where μ_{EOF} is the electroosmotic mobility of the BGE and is a constant of proportionality between the electroosmotic velocity and the electric field strength.

The EOF may also be expressed in terms of mobility by the following equation,

$$u_{EOF} = \left(\frac{\varepsilon \zeta}{4\pi\eta} \right) \tag{1.8}$$

where the electroosmotic mobility is directly proportional to the dielectic constant of the medium, ε, and the zeta potential at the capillary/buffer interface, ζ, and is inversely proportional to the viscosity, η, of the medium. The zeta potential refers to a potential difference created very close to the surface as a result of the counterions forming the electric double layer and is given by equation 1.9,

$$\zeta = 4\pi\delta e / \varepsilon \tag{1.9}$$

where δ is the thickness of the diffuse double layer, e is the charge per unit surface area, and ε is the dielectric constant of the buffer.

The zeta potential is largely dependent on the electrostatic nature of the capillary surface and less on the ionic nature of the BGE. At low pH, the EOF is suppressed due to the conversion of the SiO^- groups to SiOH resulting in a decrease in the zeta potential. At high ionic strength, the EOF decreases due to the collapse of the electric double layer [9].

One of the advantages of CZE over HPLC is the flat flow profile that originates from an electrokinetic pumping mechanism [2]. Figure 1.3 compares the flat flow profile obtained in an electrokinetic based separation technique such as CE to the laminar profile obtained in hydrodynamic technique such as HPLC.

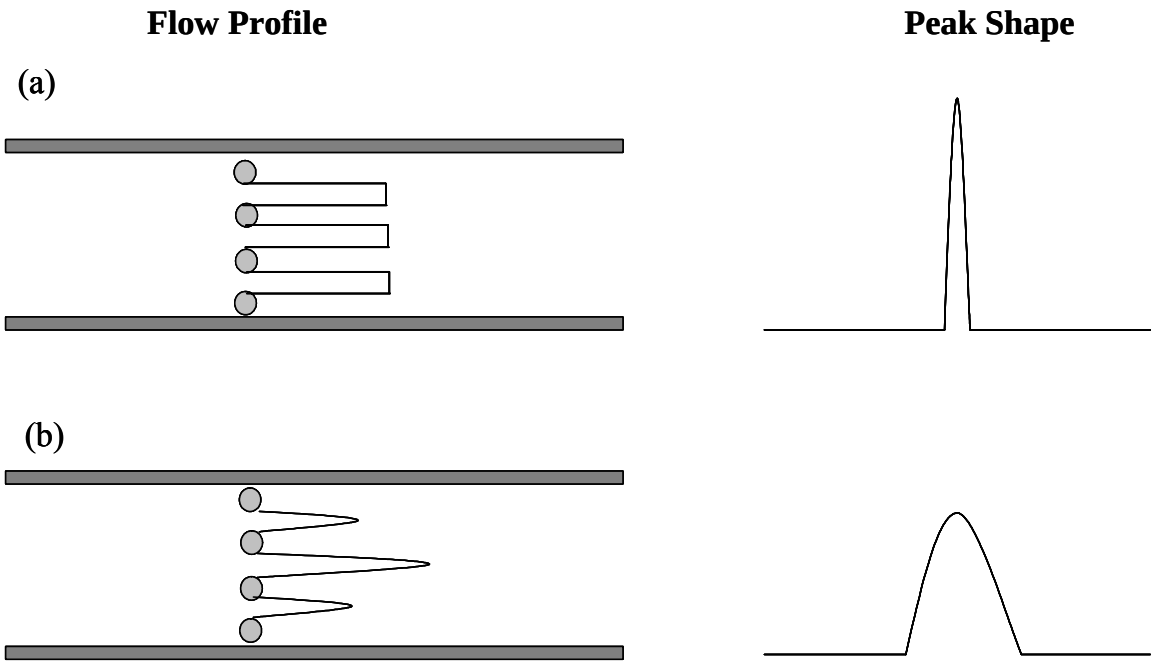

Figure 1.3 Flow velocity profiles in a packed separation column (a) An electrokinetic driven and (b) A pressure driven flow profile.

In a flat flow profile, all solute molecules move with a velocity resulting from the EOF, irrespective of the cross-sectional position in the capillary [10]. As a result, the solutes elute as narrow bands yielding high peak efficiencies. Although, frictional drag causes the EOF to decrease more at the wall than in the rest of the separation column, this does not interfere with the overall flow profile because the area near the wall is quite small. In HPLC, where a hydrodynamic flow is applied, solutes in the center of the separation column move faster than those closer to the capillary wall, as shown in Figure 1.3 (b). This, in turn, leads to broader bands with lower peak efficiencies.

The separation of cations and anions is based on differences in the apparent mobilities as well as the analyte charge-to-size ratio. The apparent mobility of an analyte, $\mu_{app,}$ is due to contributions from the electrophoretic mobility, μ_{ep}, of the analyte plus the electrophoretic mobility of the BGE, μ_{EOF} , as shown in equation 1.10.

$$\mu_{app} = \mu_{ep} + \mu_{EOF} \tag{1.10}$$

The apparent velocity, v_{app}, of an analyte is directly proportional to the electric field strength and the apparent mobility such that,

$$v_{app} = \mu_{app} E \tag{1.11}$$

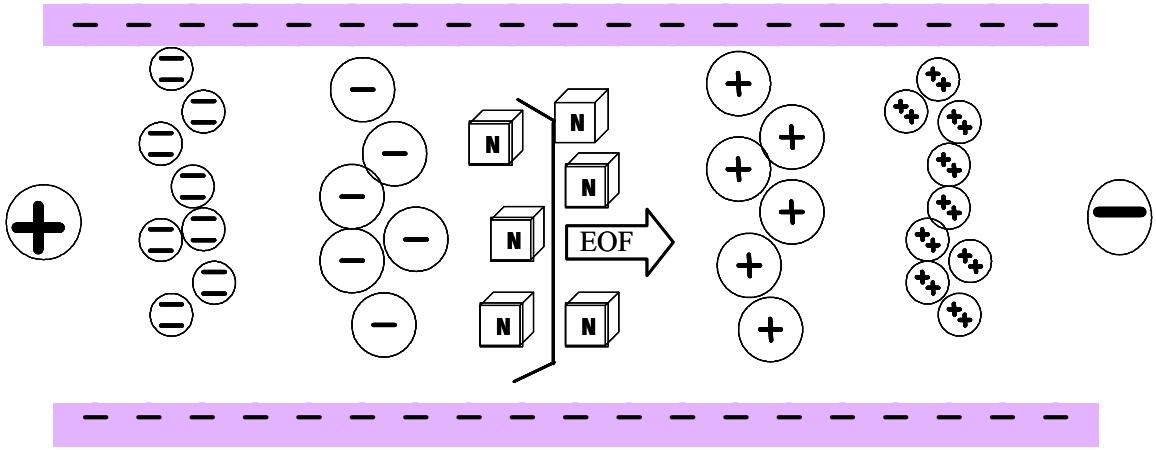

Figure 1.4 Differential elution order of cationic (+), neutral (N), and anionic (–) analytes in CZE.

When a positive voltage is applied, cations move in the same direction as the EOF towards the cathode. Thus, both μ_{ep} and μ_{EOF} have the same sign and, hence, μ_{app} is greater than μ_{EOF} in equation 1.10. As a result, cations elute before the EOF. On the other hand, anions migrate in opposite direction to the EOF on application of a positive voltage. At pH values greater than 3, the magnitude of the EOF is greater than the electrophoretic mobility of the anions, causing them to migrate to the cathode. However, for very acidic conditions where the pH is less than 3, the magnitude of the EOF is very small, and in this case anions migrate to the anode. The separation of neutral analytes is impossible in CZE because uncharged species do not possess an electrophoretic mobility, $\mu_{ep} = 0$ and they migrate in the

same direction and with the same velocity as the EOF. Figure 1.4 is an illustration of the elution order of cations, neutrals, and anions under the influence of an applied electric field in CZE.

1.3 Micellar Electrokinetic Chromatography

Micellar electrokinetic chromatography (MEKC) is one of the separation modes of CE where both neutral and charged compounds are separated by use of a micellar pseudostationary phase. A micellar pseudostationary phase is created by the addition of surfactants into the BGE at sufficiently high concentrations to ensure micelle formation. This technique was introduced by Terabe *et al*. [11] who demonstrated the separation of fourteen phenol derivatives with high efficiencies using sodium dodecyl sulfate (SDS) micelles. A more detailed discussion of micelles and polymeric surfactants as used pseudostationary phases for MEKC can be found in Section 1.3.1 and 1.3.2 respectively.

1.3.1 Surfactants and Micelles

Surfactants are amphiphilic molecules that consist of a polar head group and a long hydrocarbon (non-polar) chain referred to as the tail. They are broadly categorized according to the charge of the polar head group [12]. They can be anionic ($R\text{-}X^-M^+$), cationic ($R\text{-}N^+(CH3)_3X^-$), zwitterionic ($R\text{-}N^+(CH_3)_2CH_2X^-$) or nonionic [$R\text{-}(OCH_2)CH_2))_nOH))$], where R represents the aliphatic chain, M^+ is a metal ion, X^- is typically a halogen, carboxylate, sulfonate or sulfate, and n is an integer [13].

In the presence of water, surfactants display unique behavior in order to reduce unfavorable interactions between polar water molecules and non-polar tails [14]. At low concentrations, surfactants position themselves in such a way that the polar head group interacts with water while the non-polar tail interacts with the region above the water surface.

This in turn reduces the surface tension of water by reducing the cohesive energy of water molecules. At high concentrations, surfactant monomers can no longer position themselves in this way and spontaneously aggregate to form micelles. In polar solutions, micelles form aggregates with the head groups located on the exterior and the tails on the interior. This is because the polar head group is hydrophilic or 'water loving' and, therefore, is compatible with an aqueous environment while the tail is hydrophobic or 'water hating' and is sequestered from the polar solvent.

A dynamic equilibrium exists between surfactant monomers and the micelle and is dependent on temperature, surfactant concentration, solvent additives, and type of solvent used in dissolving the surfactant [15]. At a certain surfactant concentration, referred to as the critical micellar concentration (CMC), micelle aggregates are spontaneously formed. Figure 1.5 is a representation of the aggregation of surfactant monomers to form a micelle. Both thermodynamic and kinetic processes dictate the equilibrium shift between surfactant monomers and micelles. The aggregation number, N, of a micelle refers to the average number of surfactant monomers that form a micelle and may be calculated using equation 1.12,

$$N = \frac{[\text{Surfactant}] - \text{CMC}}{[M]} \tag{1.12}$$

where M is the molecular weight of the surfactant monomer. A micelle is typically composed of 40 to 140 molecules, and this aggregation number is dependent on the surfactant type as well as the conditions of the solvent used to dissolve the surfactant. Several analytical techniques such as light scattering [16-18], fluorescence [19-21], and nuclear magnetic resonance [21, 22] have been employed for the determination of the aggregation number

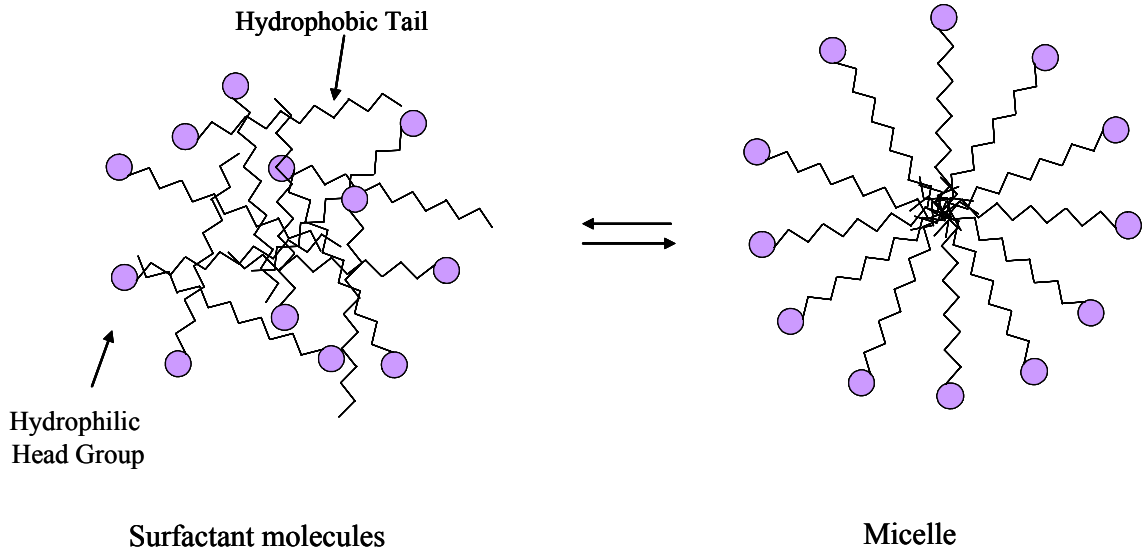

Figure 1.5 Representation of the aggregation of surfactant molecules above the CMC to form a micelle with a hydrophobic core.

The structure of a micelle is determined by both the repulsive and attractive forces originating from the hydrophilic head and hydrophobic tail groups [23]. Although micelle shape is debatable, a number of separate models on the shape of micelles have been presented ranging from a lamellar, spherical, rod shape, and disorganized as shown in Figure 1.6. McBain [24] proposed the coexistence of spherical and lamellar micelles in solutions while Dye and Anacker [25] proposed micelles as rod-shaped. Menger proposed a completely different model where micelles were depicted as disorganized with looping and non-radial distributions of chains [26]. Lastly, in Hartley's model, micelles were projected as spherical and with charged groups situated at the micellar surface [27]. Despite the variety of views on micellar shape, the Hartley model gives a successful explanation of micellar properties. In this model, the inside core of the micelle has properties of liquid hydrocarbons and thus, micelles are able to solubilize hydrophobic molecules [14, 28]. In addition, the observed drop in conductance of a surfactant solution at the CMC is attributed to counterions

being bound to the charged head groups of surfactants. The Hartley model is supported by additional studies performed using neutral small-angle scattering experiments on ionic micelles that indicate a spherical micelle shape [29-31].

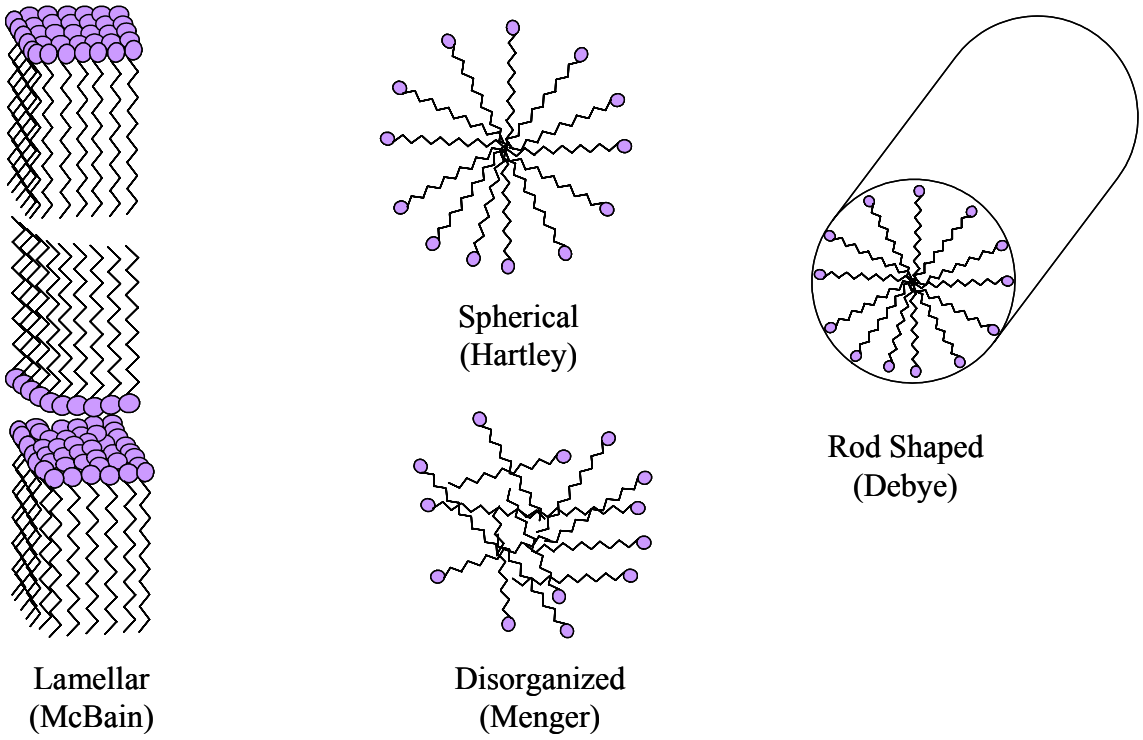

Figure 1.6 Proposed micelle shapes and structures.

Although successful separations have been accomplished by the use of conventional micelles, their application for MEKC is limited. The dynamic equilibrium that exists between surfactant monomers and micelles is a source thermodynamic instability [32]. This ultimately leads to chromatographic band broadening resulting from a reduction in the mass transfer rate between the micelles and analytes. Polymeric surfactants can be used to alleviate the problem associated with conventional micelles.

1.3.2 Polymeric Surfactants (Molecular Micelles)

Polymeric surfactants (or molecular micelles) are large macromolecules formed by the polymerization of double bonds of surfactant monomers at concentrations well above the CMC. Figure 1.7 is a representation of the structure of a typical polymeric surfactant synthesized in our laboratory. It consists of surfactant molecules with an amino acid head groups that are covalently bound at the tail.

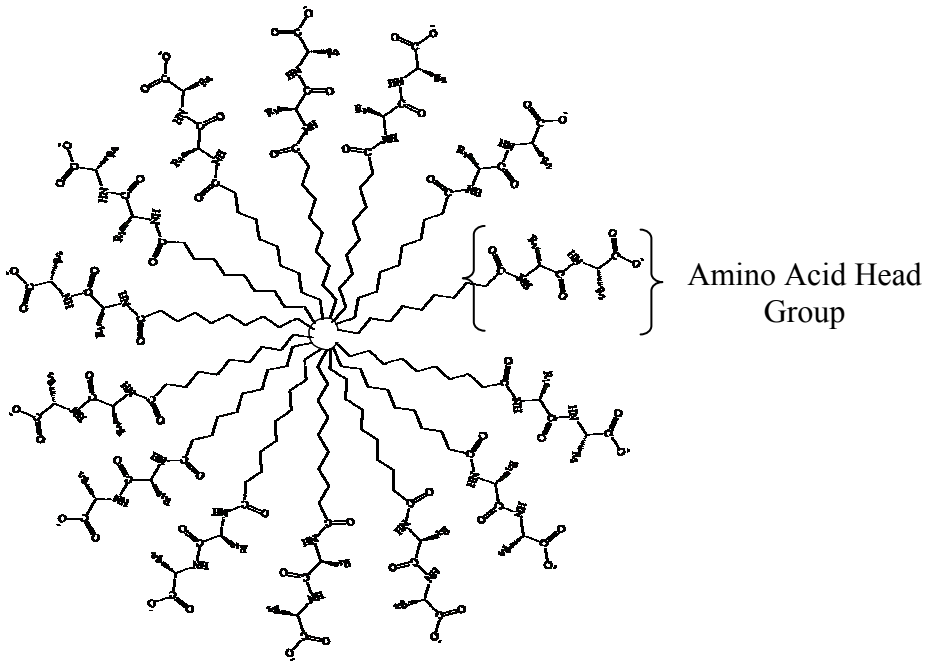

Figure 1.7 Structural representation of a dipeptide polymeric surfactant. R_1 and R_2 represent substituent groups.

The application of polymeric surfactants as pseudostationary phases for MEKC presents several advantages. First, the presence of covalent bonds between surfactant monomers eliminates the dynamic equilibrium that exists in conventional (unpolymerized) micelles resulting in enhanced stability, rigidity, and controllable sizes of the polymeric surfactant. Second, polymeric surfactants can be used at lower concentrations resulting in minimal Joule heating compared to conventional micelles that generally require concentrations of at least

two to ten times higher than the CMC. Third, higher amounts of organic modifiers in the mobile phase, necessary to enhance MEKC separations, can be used with polymeric surfactants without disrupting the micellar configuration [33, 34]. For example, approximately 65-75% of acetonitrile or methanol can be added to the BGE when polymeric surfactants serve as the pseudostationary phase in MEKC, while only 30-40% of these organic modifiers can be tolerated by SDS micelles [35-37].

Over the past years, extensive studies on the development and applications of achiral [33, 36, 38-40] and chiral [41-43] polymeric surfactants for MEKC separations have been reported. Palmer and coworkers [33, 40] were the first to introduce the use of an achiral polymerized surfactant, poly (sodium-10-undecylenate), for the separation of neutral compounds including polycylic aromatic hydrocarbons (PAHs). Although the polymeric surfactants yielded high efficiency separations of difficult to separate compounds, the presence of the carboxylated head groups limited the surfactant's solubility under acidic conditions. In addition, non-uniform migration times and cloudiness in the anodic buffer vials after a few were reported [40]. Further developments to alleviate these problems were achieved by the synthesis of the polymeric surfactant, poly (sodium undecenyl sulfate), poly-SUS, consisting of a sulfonate head group [36, 38, 39]. The use of chemical ionization for surfactant polymers has been indicated to result in lower yield and contamination of polymeric surfactant [37]. By use of γ-irradiation for surfactant polymerization the Warner group was able to achieved high poly-SUS yield with minimal contamination (97-99% purity) [36].

Polymeric surfactants have been applied for efficient chiral separations. In 1994 Warner and Wang [44] reported the synthesis and application of a single amino acid based polymeric

surfactant, poly (sodium undecenyl-L-valinate), poly-L-SUV, for the separation of 1,1′-bi-2-naphthyl-2,2′-dihydrogen phosphate and D,L-laudanosine. Further studies extending the range of chiral analytes were explored by Dobashi *et al*. [45] and our research group [46]. Shamsi *et al*. [47] compared the chiral recognition ability of poly-L-SUV containing a single amino acid head group with that of poly (sodium undecenyl-L-valine valinate), poly-L-SUVV, a polymeric surfactant containing a dipeptide head group for the separation of acidic, basic, and neutral analytes. Chiral selectivity was significantly improved by use of poly-L-SUVV in the separation of basic and acidic compounds; however, poly-L-SUV resulted in better resolution of the neutral analytes but with lower peak efficiency and a longer migration time.

The next logical progression of these studies to elucidate the interactive effect observed between analytes with single amino acid or dipeptide polymeric surfactants was initiated by Billiot *et al*. [41-43, 48]. The depth to which an analyte penetrated into the hydrophobic core of the polymeric surfactant and amino acid order on the dipeptide polymeric surfactant were found to be major factors influencing chiral selectivity. Electrostatic, hydrophobic, and steric hindrance interactions governed analyte penetration. In addition, it was observed that for dipeptide surfactants, although the enantiomer preferentially associated with one of the chiral centers, the interaction was not necessarily limited to that chiral center [43]. Based upon these observations, Shamsi *et al*. [49] were able to select poly (sodium undecenyl-L-leucine valinate), poly-L-SULV, a versatile chiral selector for a large number of chiral analytes. Poly-L-SULV was capable of separating a total of 58 out of 75 racemic compounds at optimal MEKC separation conditions. The chiral resolution success rate for cationic and neutral compounds was found to be 77% and 83% respectively; however, the authors found it difficult to resolve anionic analytes.

Studies of the combined use of polymeric surfactants with cyclodextrins with an aim of enhancing chiral selectivity have been reported [50-52]. The design of novel polymeric surfactants for increased selectivity and for separation of particular groups of analytes is ongoing [53-56] and has yet to be fully exploited.

1.3.3 Micellar Electrokinetic Chromatography Theory

One of the advantages of the use of micelles for separations in CE is that they enable the separation of neutral compounds which is not possible with CZE. The micelles in solution form a pseudostationary phase into which all analytes partition. Separation is based on the relative affinity of an analyte for the hydrophobic interior and/or hydrophilic exterior of the pseudostationary phase. Figure 1.8 is a representation of the partitioning of analytes with micelles in the mobile phase in MEKC.

In an uncoated fused-silica capillary, anionic micelles migrate toward the anode under the influence of a positive voltage. Although the direction of the anionic micelles is opposite to that of the EOF, the anionic micelles are carried towards the cathode because the magnitude of the EOF is generally greater than the electrophoretic velocity of the micelles. If cationic micelles are used as a pseudostationary phase, the direction of the EOF is reversed because the positively charged micelles will dynamically coat the negatively charged fused-silica wall.

In MEKC analytes are separated based on their differences in the rates of partitioning between the stationary phase and the mobile phase [57].

Equation 1.13 represents the equilibrium process of an analyte that occurs in MEKC between the mobile phase and the pseudostationary phase.

$$\text{Mobile Phase} \leftrightarrow \text{Pseudostationary Phase} \qquad (1.13)$$

The partition coefficient (K) is the equilibrium constant for this equation and is used to evaluate column effectiveness in separating analytes. It is given by equation 1.14,

$$K = \frac{c_S}{c_M} \tag{1.14}$$

where c_S is the molar concentration of the analyte in the pseudostationary phase and c_M is the concentration of analyte in the mobile phase.

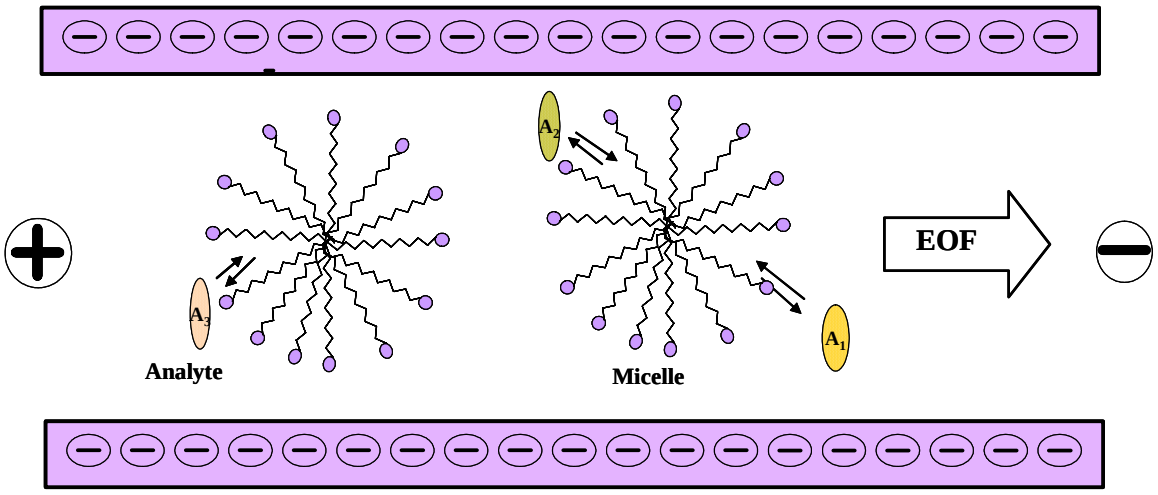

Figure 1.8 Representation of the partitioning of analytes into a pseudostationary phase in MEKC.

The rate of migration of analytes depends on the partition coefficient between the micelle and the mobile phase and is represented by the retention factor, k', given by equation 1.15,

$$k' = \frac{t_r - t_0}{t_0\left(1 - \frac{t_r}{t_{mc}}\right)} \tag{1.15}$$

where t_r and t_0 are the retention times of the analyte and the neutral marker respectively, and t_{mc} is the migration time of the micelle. In the case where t_{mc} approaches infinity, the equation above becomes similar to the conventional chromatographic equation for k'.

$$k' = \frac{t_r - t_0}{t_0} \tag{1.16}$$

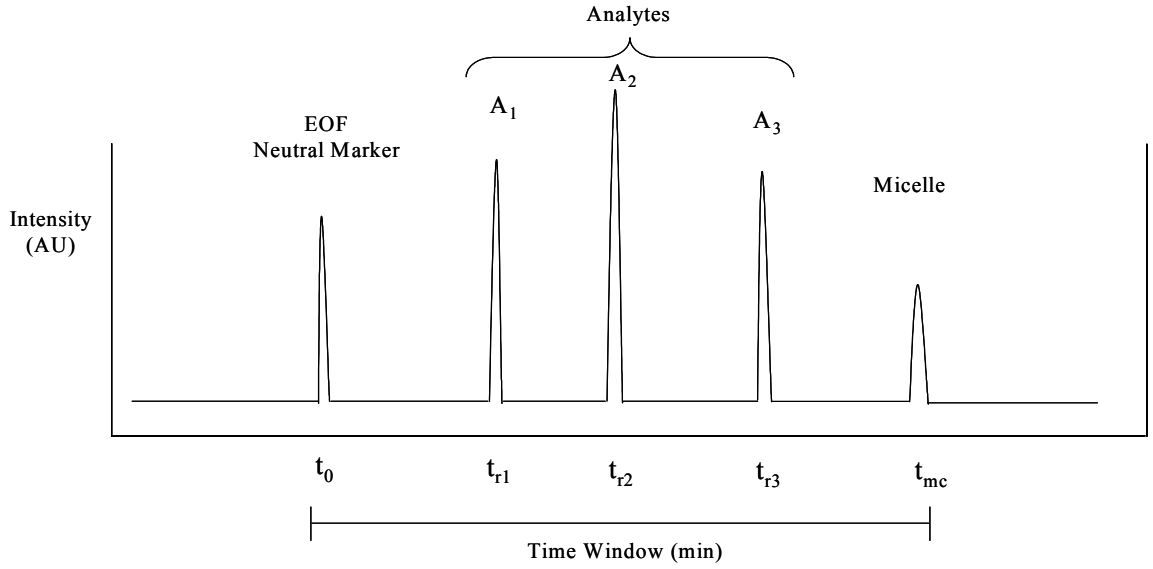

Figure 1.9 Representation of the elution window in MEKC.

Figure 1.9 illustrates the time window for the elution of analytes in MEKC. Analytes that have minimal interactions with the pseudostationary phase elute at a faster rate than hydrophobic analytes or analytes which have a strong affinity to the pseudostationary phase. Selectivity, α, is used to describe how well the separation column distinguishes between the types of analytes. In the separation of two analytes, α, is defined as the ratio of their retention factors,

$$\alpha = \frac{k'_2}{k'_1} \tag{1.17}$$

such that k'_1 and k'_2 are the first and second eluting analytes respectively. Thus, α is always greater than unity.

Selectivity can also be calculated based on the retention times of the analytes.

$$\alpha = \frac{t_{r2} - t_0}{t_{r1} - t_0} \tag{1.18}$$

The resolution, R_s, describes the separation distance between two analytes and can be computed using equation 1.19,

$$R_S = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{k'_2 + 1}\right)\left(\frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 - \left(\frac{t_0}{t_{mc}}\right)k'_1}\right) \tag{1.19}$$

where N is the number of theoretical plates and is a measure of the peak efficiency. From equation 1.19, it should be noted that the highest resolution is obtained when there is a large difference in time between t_0 and t_{mc}. In addition, it is clear from equation 1.19 that resolution is a function of efficiency, selectivity, and retention.

1.4 Capillary Electrochromatography

Capillary electrochromatography (CEC) is a mode of CE where a stationary phase in various formats is incorporated into the fused-silica capillary. The technique is a hybrid of CZE and HPLC and thus combines the high selectivity of HPLC and the high peak efficiency of CE. Like CE, an electric field is applied across the separation column generating EOF that carries the mobile phase and solutes through the column resulting in high peak efficiency separations. Similar to HPLC, neutral analytes can be separated by partitioning between the mobile and stationary phases.

Although CEC is similar in some aspects to both CZE and HPLC, CEC presents some advantages over these techniques that have lead to its popularity. Contrary to CZE, CEC is capable of separating difficult analytes, such as neutral analytes, because it incorporates a stationary phase into which neutrals partition. In addition, the stationary phase can be tailored

in order to increase specific interactions with certain analytes that CZE cannot separate, particularly chiral molecules. Compared to HPLC where a mechanical pump is used to drive the flow, CEC uses an applied electric voltage to propel the mobile phase. This in turn results in higher peak efficiencies of up to 300 000 and thus, higher resolution [58]. Unlike HPLC, it is possible to increase the column length in packed columns in CEC even with particle sizes as small as 1 μm due to the electrically driven flow that eliminates the pressure drop inherent in HPLC [59].

The concept of CEC was first introduced by Pretorious *et al*. [60] who proposed the use of an electrokinetic pumping mechanism as an alternative to a hydrodynamic pumping mechanism for separations. Almost a decade later the theory of CEC was demonstrated by Jorgenson and Lukacs [61] and this led to an increase in CEC attention. In their studies, they demonstrated the separation of 2-methylanthracene and perylene with good efficiencies using a 170 μm internal diameter capillary packed with octadecyl silica [3]. Further developments on applications of CEC were investigated by Knox and Grant [62-64], and from that time CEC has continued to grow extensively.

The separation of solutes in CEC is based on two components: (1) the distribution ratio (a chromatographic factor) and (2) the differential migration (an electrophoretic factor). The distribution ratio is a result of the differential interactions between the stationary phase and the mobile phase, while the differential migration arises from the electrophoretic mobility of the analyte. For neutral analytes the electrophoretic mobility is zero and the separation is based on the differential interaction with the stationary phase; however, the elution is driven by the EOF. For charged analytes, the separation is based on both the electrophoretic mobility and the distribution ratio.

The retention factor, k'_{CEC}, in CEC incorporates both the chromatographic and electrophoretic factor. It may be computed in different ways [65-67] and one method is summarized by equation 1.20,

$$k'_{CEC} = \frac{k' - \left(\frac{\mu_{ep}}{\mu_{EOF}}\right)}{1 + \left(\frac{\mu_{ep}}{\mu_{EOF}}\right)} \tag{1.20}$$

where k' is the retention resulting from the chromatographic factor. In the case of neutral analytes, μ_{ep} is zero, reducing the equation to k'_{CEC} equals k'. This implies that neutral analytes are separated by a purely chromatographic mechanism. However, in the case of charged analytes both chromatographic and electophoretic mechanisms contribution to the separation.

There are three types of CEC: packed column CEC (PC-CEC), monolithic columns, and open-tubular CEC (OT-CEC) [68]. Figure1.10 is an illustration of the differences in spatial location of stationary phase in a fused-silica capillary for these three modes.

In PC-CEC, the stationary phase is packed in a fused-silica capillary with an internal diameter of 50-100 μm. Typically, the stationary phase consists of 3-5 μm C_{18} or C_8 silica supported in the capillary by two retaining frits. The packing procedure involves first preparing a frit by burning the silica capillary and then pumping a slurry of the stationary phase into the capillary under high pressure. Thereafter, another retaining frit is made and a detection window is positioned adjacent to frits in order to minimize postcolumn band broadening [59].

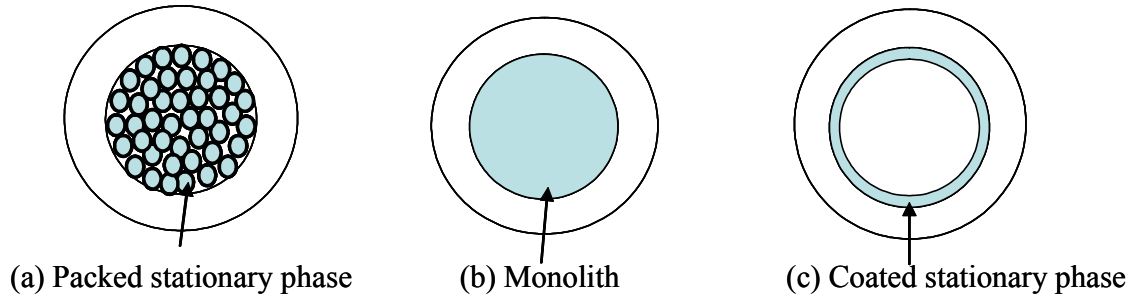

Figure 1.10 Schematic representation of the types CEC stationary phases in (a) packed-column CEC (b)monolith CEC (c) open-tubular CEC. Modified from reference [69].

There are a number of drawbacks associated with PC-CEC that limit its practical application. The fabrication of stable frits that retain the packing material and maintain an unrestricted flow is extremely difficult. Formation of bubbles around the packing material and frit often occurs resulting in unstable baselines, current breakdown, and irreproducible elution times. In addition, the narrow inner diameter of capillaries used in PC-CEC makes the packing procedure difficult relative to the packing of HPLC columns. These difficulties have led to the use of monolithic columns as a suitable alternative to PC-CEC.

In monolithic CEC, columns consist of a continuous unitary macroporous structure or monolith prepared by either an *in situ* polymerization of an organic moiety or sol-gel material [70-73]. There are three main types of monolithic columns: organic porous, silica sol-gel, and immobilized particles. Organic porous monoliths are prepared by the polymerization of monomers in the column using free radical, thermal, or UV initiation. In silica sol-gel monoliths, the capillary is filled with monomers that undergo hydrolysis and polycondensation reactions to form a porous silica gel network. In contrast, immobilized

particle monoliths, in either an organic polymer or sol-gel matrix, are prepared by first packing stationary phase particles using temporary retaining frits. Thereafter, a mixture of organosilanes or a methacrylated-based monomer and a porogenic solvent is pumped through the column for polymerization.

The advantage of using monolithic columns in CEC is that the surface of the monolith may be functionalized to provide the desired chromatographic properties. In addition, the synthetic procedure is relatively simple and does not require the fabrication of frits as in PC-CEC.

OT-CEC is also considered a suitable alternative to PC-CEC [74]. In OT-CEC the stationary phase is immobilized onto the surface of the capillary by covalent bonding or physical adsorption, and the columns are categorized into sol-gel [75, 76], molecular imprinting [77, 78], etching and chemical bonding [79-81], porous silica layers [82-84], and adsorbed coatings [65, 85, 86]. The advantage of OT-CEC is that typical columns of 10 to 25 μm internal diameter are used leading to higher separation efficiency relative to PC-CEC where band-broadening results from eddy diffusion in the column packings and the frits. In addition, the smaller capillary diameters used in OT-CEC affords the application of high voltages with minimal Joule heating. However, it should be noted that OT-CEC has its own limitations. For example, the presence of a low phase ratio often results in lower peak resolution as compared to PC-CEC. In some cases peak tailing resulting in analyte adsorption onto the capillary wall is observed. Due to the small diameter in OT-CEC, analyte injection size is very low, that is, in the nL or pL range.

The following section is a detailed discussion of polyelectrolyte multilayer (PEM) coatings, a type of OT-CEC investigated in this dissertation.

1.4.1 Fundamental Aspects of Polyelectrolyte Multilayers

Decher *et al*. [87, 88] demonstrated that a stable polyelectrolyte multilayer (PEM) could be prepared by the alternate exposure of a surface to oppositely charged polyelectrolyte solutions. Each polymer deposition cycle imparts a reproducible amount of polymer and reverses the surface charge leaving the surface primed for the next oppositely-charged polymer layer. The formation of PEMs occurs via an ion exchange mechanism where there is an *in situ* displacement of counterions in the polymer solutions by the charged polymer segments according to equation 1.21 [89],

$$Pol^{-}M^{+}_{(m)} + Pol^{+}A^{-}_{(aq)} \leftrightarrow Pol^{-}Pol^{+}_{(m)} + M^{+}_{(aq)} + A^{-}_{(aq)} \tag{1.21}$$

where Pol^{+} and Pol^{-} are the polymer charged segments, M^{+} and A^{-} are the salt counterions, and *m* refers to the region closest to the surface. Figure 1.11 is a representation of a PEM deposited onto the surface of a fused-silica capillary with hypothetical cationic and anionic polymers.

The growth process of a PEM is due to two important properties: the overcompensation of surface charge and reversal of charge after each deposition cycle [90]. Subsequent adsorption of a new polymer layer onto the preceding layer is possible because the excessive charge resulting from the previous polymer layer. The net charge of the multilayer is generated by the last layer deposited; hence, it is theoretically possible to build an infinite number of layers [91]. Stability of the polymer layer is a result of the electrostatic interactions between multiple layers.

Although the formation of PEMs is simple, the internal structure, which has been probed by various techniques including neutron reflectometry [92], atomic force microscopy [93], and solid state NMR [94], reveals considerable complexity. The layers deposited consist of

stratified structures in which polyanions and polycations of individual layers interpenetrate each other. One layer may be dispersed three or four layers from its original location [90]. The interpenetration is a result of the intrinsic structure of the charge compensation with multilayers [89]. In addition to layer interpenetrations, surface roughness and morphology have been shown to be influenced by salt added to one or both polymer solutions [93].

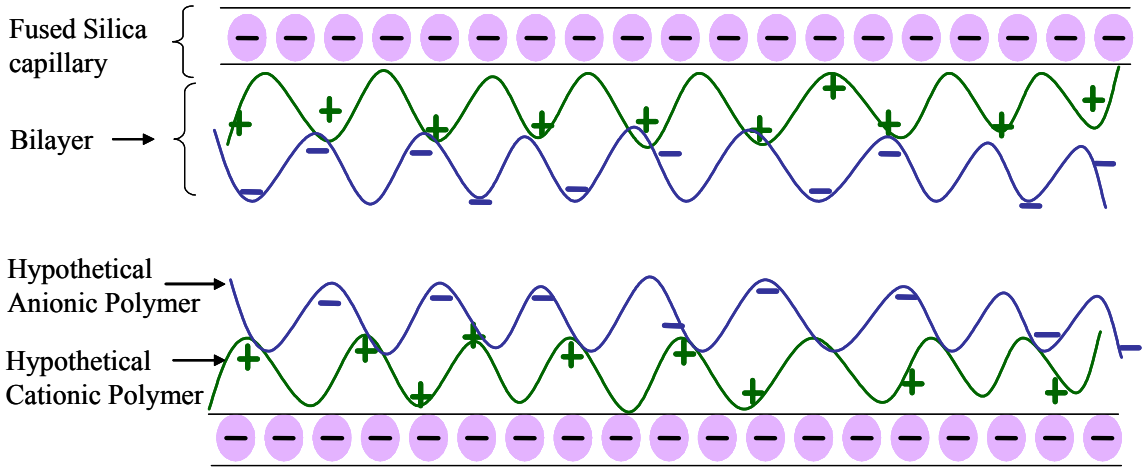

Figure 1.11 Hypothetical representation of a polyelectrolyte multilayer coating.

There are a number of properties of PEM coatings that ultimately affect the chromatographic performance of coated surfaces. For example, the thickness of the PEM is an important factor that has been shown to affect the resolution and peak efficiency of analytes [95, 96]. The number of bilayers is also one of the main factors that affect PEM thickness. A bilayer, or a layer pair, refers to a positive and negative layer of polymer. An increase in the number of bilayers increases the thickness. It has been shown that the layers deposited closer to the substrate have significantly smaller thickness compared to those deposited far from the substrate [93, 97]. This non-linear growth of the initial layers is

attributed to a roughening of the polymer layer interfaces that produces a larger number of adsorption sites for deposition of subsequent layers [89, 92].

Another significant factor that influences film thickness is the concentration of salt in the polymer deposition solution. A linear relationship has been established between the thickness of the film and the salt concentration. However, this relationship is dependent on the type of polyelectrolyte pair used in building the PEM [98]. For example a study by Graul and Schlenoff [99] demonstrated that the dependence of the thickness of a layer pair of cationic poly (diallyldimethylammonium chloride) (PDADMAC) and anionic polystyrene sulfonate (PSS) on molar salt concentration to be different from that of a layer pair of poly (allylamine hydrochloride) (PAH) and PSS determined by Losche *et al.* [92] .

The surface charge of the PEM is another important property affecting chromatographic performance. This is because the charge of the PEM determines the direction and magnitude of the EOF. A normal EOF is obtained when the exposed layer is negatively charged and a reversal of EOF occurs when the exposed layer is positively charged. It has been shown that surface charge is determined by the concentration of salt ions dissolved in the polyelectrolyte solutions and is independent of film thickness [99, 100]. Radiochemical methods have been developed to measure the magnitude of this excess surface charge [90, 101].

1.4.2 PEMs Used in Open-Tubular Capillary Electrochromatography

The application of a PEM coating for separations in OT-CEC was first introduced by Katayama *et al*. [102, 103] in 1998. Fused-silica capillaries were modified using a simple coating procedure referred to as the successive multiple ionic-polymer layer (SMIL) coating. In an initial study [102] the cationic polymer, polybrene, was sandwiched between the anionic polymer, dextran sulfate (DS) and the uncoated fused-silica capillary by non-covalent

bonding. This coating was very stable, tolerant to organic solvents, 1M NaOH, and SDS surfactant. In addition, the coating exhibited a pH-independent EOF from anode to cathode in the pH range of 2 to 11. The efficient separation of acidic proteins under physiologic conditions was possible since the coating minimized wall adsorption. The simultaneous separation of cationic, anionic, and neutral amino acids was also achieved in this study.

In another study [103], the SMIL coating was prepared by first coating the inner wall with polybrene, followed by DS and finally polybrene. The coating endured more than 600 replicate analyses demonstrating good reproducibility and run-to-run and capillary-to-capillary with a relative standard deviation (RSD) of the EOF of less than 1%. In addition, the coating exhibited remarkable stability against 1 M NaOH and 0.1 M HCl. The separation of basic proteins resulted in good performances even when performed at the isoelectric points of the proteins.

Graul and Schlenoff [99] reported a similar coating procedure where alternating layers of PDADMAC and PSS were deposited on the surface of fused-silica capillaries. Separation of proteins, with good efficiencies, was obtained due to the suppression of adsorption on the wall as a result of electrostatic repulsions. Improved resolution of proteins was observed when a 6.5 layer pair PEM column was used relative to a column consisting of a single layer of PDADMAC. However, a decrease in peak efficiency was observed in the separation of neutral analytes such as fluorobenzene, phenol and p-cresol that partitioned into the PEM coating. The stability of the EOF at extreme pH, ionic strength, dehydration and rehydration was quite impressive.

1.4.2.1 PEMs Incorporating Polymeric Surfactants

The presence of negatively charged hydrophilic groups on polymeric surfactants enables their ready integration into PEMs. In such use, polymeric surfactants may be applied in OT-CEC for the separation of neutral analytes that will partition into the stationary phase. Recently PEMs consisting of polymeric surfactants have been reported by Warner and coworkers for achiral [104, 105] and chiral separations [95, 96]. Kapnissi *et al*. [104] reported the use of a 10 bilayer coating consisting of PDADMAC, as the cationic polymer, and poly (sodium N-undecenyl-L-glycinate), poly-SUG, as the anionic polymeric surfactant. In this study, the performance of the coating was evaluated by the separation of a series of benzodiazepines. The coating exhibited remarkable stability, even after being exposed to extreme pH conditions, and endured more than 200 runs under normal conditions. In addition to stability, the run-to-run, day-to-day, week-to-week, and capillary-to-capillary RSDs of the EOF were found to be less than 1% in all cases. The importance of the use of polymeric surfactants in the PEM coating was investigated by comparing the separations obtained with the monomeric (unpolymerized) micelles. No separation was observed in the latter case due to the dynamic equilibrium between monomers and micelle aggregates.

Kamande *et al*. [105] investigated the separation of both phenols and benzodiazepine analytes using a 1-bilayer coating consisting of cationic PDADMAC and poly (sodium undecenyl sulfate), poly-SUS, as the anionic polymeric surfactant. The coating showed good selectivity for the analytes investigated. The chromatographic performance of the PEM-coated capillaries was compared to that using MEKC and CZE. An improvement in resolution and selectivity was shown in the PEM-coated capillaries. The stability of the coating was good, enduring over 100 runs.

1.4.2.2 Chiral Separations with PEMs

Chiral separation of enantiomeric drugs is of interest because of the variation in biological activities exhibited by the enantiomers. Mayer and Schurig were the first to report enantiomeric separation in OT-CEC using capillaries coated with immobilized Chiralsil-Dex [106]. Thereafter, Rmaile and Schlenoff [107] reported the use of optically active PEMs for the chiral separation of ascorbic acid, 3-3(3,4-dihydroxyphenyl)-L/D-alanine and a chiral viologen. The PEMs were constructed using polypeptides, the L- and D- forms of poly (lysine) and poly (glutamic acid). A 16-layer PEM was constructed for the separation of ascorbic acid in OT-CEC yielding theoretical plates of 46 000 and 29 000 for the L- and D-enantiomer, respectively. The chirality of the PEM coating was found to be selective for a particular enantiomeric analyte. For example, the PEM constructed from the D- forms of poly (lysine) and poly (glutamic acid) was more selective for the D- enantiomer studied. In addition, it was found that the use of two optically active polyelectrolytes in the PEM coating yielded a higher chiral selectivity than the use of only a single optically active polyelectrolyte.

In another study, Kapnissi *et al*. [95] investigated the use of PEMs consisting of the chiral poly-L-SULV and PDADMAC for chiral separations. The authors obtained chiral separation of five analytes, 1,1′-binaphthyl-2,2′-dihydrogenphosphate (BNP), 1,1′-bi-2-naphthol (BOH), secobarbital, pentobarbital, and temazepam. In order to achieve chiral separations, the authors modified their previous achiral PEM coating procedure [104] by reducing the number of bilayers and using additives in coating solutions. Parameters such as salt concentration, solvent additives in the polymer deposition solution, column temperature, and number of bilayers were varied to obtain optimal conditions. The coating provided excellent

reproducibility of the EOF and was found to be remarkably robust with a performance of more than 230 runs.

Kamande *et al*. [96] recently investigated the use of the chiral polypeptide, poly (L-lysine), as an alternative cationic polymer to the achiral PDADMAC. The PEM coating, consisting of the anionic poly (sodium undecenyl-L-leucine alanate) and the cationic poly (L-lysine) was applied toward the separation of BNP, BOH, 1,1′-binaphthyl-2,2′-diamine (BNA), labetalol, and sotalol. A comparison of separations using poly (L-lysine) as the cationic polymer in place of PDADMAC showed higher selectivity using the former. The number of bilayers was shown to have a significant effect on resolution and selectivity of BNP. Run-to-run and capillary-to-capillary reproducibilities of the EOF were less than 1% RSD. In addition, the coating was found to be very stable, enduring more than 290 runs.

1.4.2.3 PEMs Combined With Micellar Electrokinetic Chromatography

Bendahl *et al*. [108] illustrated the use of capillaries coated with a bilayer of polybrene and PVS, for successful MEKC separations of alkaline and neutral compounds. The stability of the coating in the presence of SDS enabled rapid MEKC separations at low pH. A pH independent EOF, in the pH range of 2 to 10, was obtained as a result of the strongly acidic PVS layer. The separation of alkaline compounds gave reproducible run-to-run and capillary-to-capillary migration times less than 1% and 2% RSD, respectively. Pranaityte *et al*. [109] investigated the use of a bilayer coating consisting of PDADMAC and PSS for MEKC separations using SDS as the pseudostationary phase. A very stable, pH independent cathodic EOF was achieved. Based on their results, the second layer of PSS was replaced by SDS micelles during flushing with the micellar electrolyte. Thus it was not necessary to add SDS to the BGE in order to achieve stable and pH independent EOF.

Finally, it is worth mentioning that PEMs have found applications in other analytical separation techniques such as microfluidic devices [110-113]. Such devices are often made of plastic substrates which suffer from a variation surface charge even from polymer to polymer. This ultimately results in an irreproducible EOF even from devices made from the same polymer substrate. The use of PEMs is advantageous in this case as it affords some degree of control of the flow and direction of EOF as well as the uniformity and distribution of surface charge.

1.5 Chirality

Chiral molecules are compounds that rotate plane polarized light and are, therefore, said to be optically active [114]. In 1843, Louis Pasteur [115], a French chemist and microbiologist, discovered the chiral properties of assymetric molecules. Since he visually separated chiral crystals, these studies were the first on chiral separations. As he was working with crystals of sodium ammonium tartrate, he noticed that some were "right-handed" while others were "left-handed". Pasteur observed that a solution of the "right-handed" crystals rotated plane polarized light in a clockwise direction, while a solution of the "left-handed" crystals rotated plane polarized light in an counterclockwise direction. Such optical activity was observed only in solution and, thus, Pasteur proposed that optical activity is a property of molecules and not crystals [116]. Using a pair of tweezers and a hand lens, he carefully separated the two kinds of crystals.

Chirality is a term used to describe the geometric property of organic molecules in which chiral molecules are non-superimpossable on their mirror image. Such compounds are called enantiomers or optical isomers and they contain either an asymmetric element in the form of either a tetrahedral carbon atom bonded to four different substituents or a plane of

asymmetry. Enantiomers have identical physical properties in an achiral environment such as solubility or boiling and melting points; however, they differ in their optical rotation of light as noted by Pasteur. The degree of optical rotation is the same for enantiomers; however, the opposite of is different and can be measured using a polarimeter. Enantiomers that rotate light to the left direction are said to be levorotatory (L) and have a negative (–) symbol, while those that rotate light to the right are said to be dextrorotatory (D) and have a positive symbol (+). While the "L or D" notation is used to label enantiomers based on their optical activity, the R and S notation is used to describe the stereochemical configuration around the asymmetric carbon. The R configuration indicates a chiral center whose priority of substitutes is in a clockwise direction while L configuration indicates a chiral center whose priority of substitutes are in an counterclockwise direction [117]. A racemic mixture consists of an equal amount of each enantiomer and is optically inactive. Such mixtures do not rotate plane polarized light because for every molecule that rotates light in one direction there another molecule that rotates light in the opposite direction.

It is well known that the enantiomers of a particular drug compound often exhibit different pharmacokinetic properties. While one enantiomeric form may produce a desired physiological effect, the other may produce no physiological effect or even be toxic. The observation was made in 1956 when the drug thalidomide was approved as a sedative and was marketed as a racemic mixture for use in Europe, Canada, and later in the United States. While the dextrorotatory enantiomer had strong sedative properties, the levorotatory enantiomer was highly teratogenic causing birth defects in babies whose mothers were administered the drug in the early stages of pregnancy. It was eventually determined that the dextrorotatory enantiomer resulted in mild teratogenic activity and the drug racemized when

administered in the human body [114]. Prompted by these observations, the Food and Drug Administration issued a policy statement requiring pharmaceutical companies to evaluate the enantiomers of a drug and employ separation techniques that discriminate between the enantiomers of a chiral drugs [118, 119].

Several analytical techniques have been developed for the separation of chiral compounds including HPLC, GC, CEC, and MEKC [120-123]. In such techniques a chiral selector in the form of an immobilized stationary phase or a BGE additive is used to provide enantioselective interactions with drug compounds resulting in separation. Hydrophobic and electrostatic interactions occur between the hydrophobic and polar parts of the chiral selector, respectively. Additional interactions such as hydrogen bonding, Van der Waals and ion-dipole interactions may result. Although the detailed mechanism of such enantioselective interactions is not clearly understood due to it complexity and multiplicity of interactions, the "three point rule" proposed by Easson and Stedman [124] has been widely used to explain this mechanism. In this rule, a minimum of three simultaneous interactions are required between the chiral selector and at least one of the enantiomers for chiral recognition to occur. In addition, at least one of the interactions has to be stereochemically dependent. The other enantiomer does not achieve the three interactions with the chiral selector due spatial restrictions and, thus, this difference in interactions between the two enantiomers results in a separation.

Part 2 Introduction to Equilibrium Gradient Techniques

1.6 Equilibrium Gradient Techniques

An equilibrium gradient method as defined by Giddings and Dahlgren [125] is "..a method in which a gradient or combination of gradients causes each species to seek an

equilibrium position along the separation path." In such a method, the analyte experiences a net force induced by an external field that results in a consistent change in the analytevelocity and direction at a particular point along the separation channel. The net force can be manipulated such that the analyte's net velocity is zero at a unique point where it is focused, leading to a concentration enhancement [126]. Thus, regardless of the initial position of the analyte along the separation channel, the analyte will be drawn into its unique focusing (equilibrium) point where its net mobility is zero [127]. Figure 1.12 is an illustration of the concentration enhancement of an analyte at the focusing point. The separation of analytes occurs as a result of the differences in analyte properties such as the electrophoretic mobility or retention, and this causes analytes to be focused at separate distinct points.

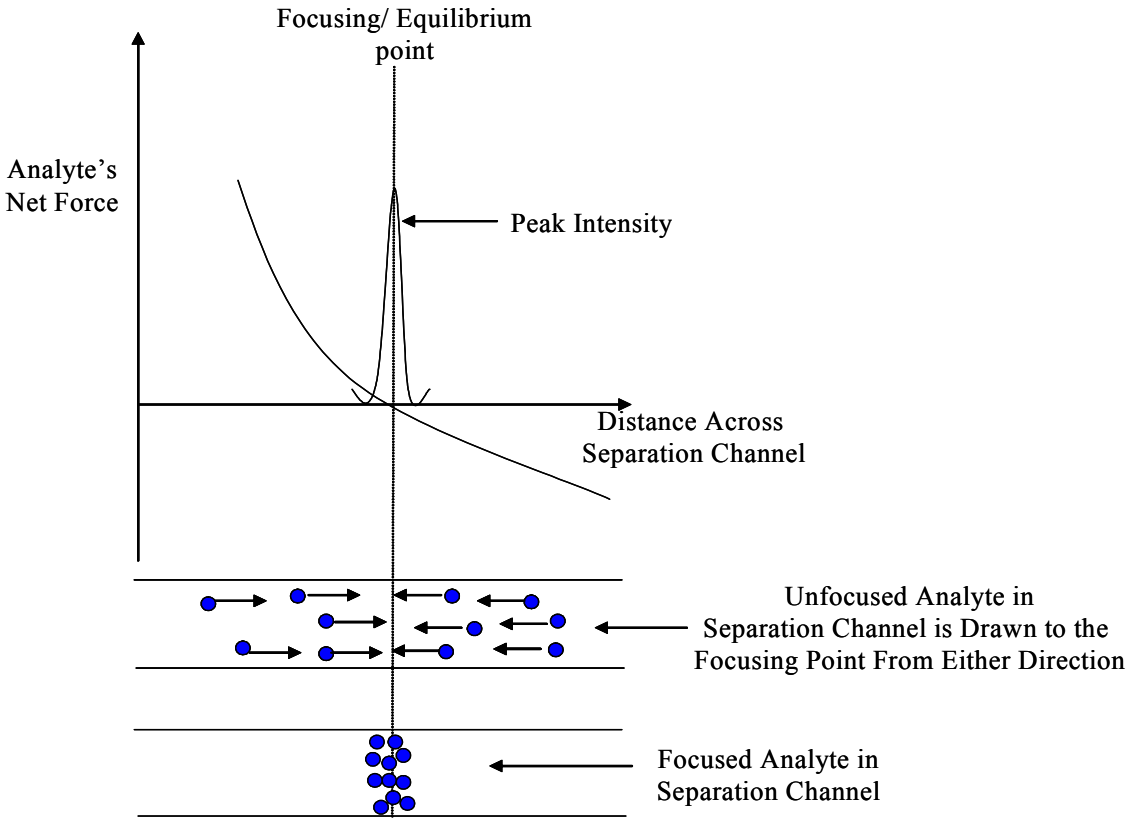

Figure 1.12 Schematic representation illustrating principle of an equilibrium gradient technique. Modified from [128].

There are several advantages associated with the application equilibrium gradient methods for separations over conventional transient separations such as CE. In equilibrium gradient techniques there is a concentration enhancement of analytes with time. Thus, lower detection limits can be achieved contrary to transient separations where peak intensities decrease with time. This in turn makes equilibrium gradient separations suitable for clinical analysis where the analyte of interest is usually at very low concentrations.

Another advantage of such separations is that analyte injection may be performed on either end of the separation channel. This is because analytes eventually migrate to their respective zero velocity point regardless of their initial position in the separation channel. In addition, injection size in equilibrium gradient separations is not as critical as in transient separations where large sample sizes result in band broadening. Dispersion effects in transient separations result as the analyte travels through the capillary. Finally, equilibrium gradient separations are more suitable for miniaturization because separation occurs at static points along the channel contrary to transient separations where longer capillaries are needed to obtain equivalent or better resolution to those obtained in separation channels.

Several equilibrium gradient techniques have been applied for focusing of analytes [128]. In general, these techniques employ a velocity gradient along the separation channel that can be applied by varying parameters such as BGE pH, electric field, temperature, and retention factor in the case where micelles are added to the mobile phase. The following sections discuss these techniques in detail.

1.6.1 Capillary Isoelectric Focusing

Capillary isoelectric focusing (CIEF) is a technique in which charged analytes are concentrated and separated along a pH gradient based on their isoelectric point (p*I*) [129]. At

the p*I* of an analyte, the negative and positive charges are balanced causing the analyte to possess a zero net charge and thus stop migrating. The pH gradient is created by use of a series of zwitterionic ampholytes with p*I* values within the desired pH range. A basic solution is applied at the cathode while an acidic solution is applied at the anode. On application of an electric field the analytes migrate to discrete points at which they are neutral which is their respective p*I* points. At this point the analytes are said to be focused, forming sharply defined analyte bands characteristic of CIEF. The analytes can be detected at their point of focus or by application of pressure or voltage through the capillary that mobilizes analytes to the detection point [130]. In CIEF the magnitude of EOF is reduced or eliminated because it may rinse out the ampholytes before focusing is achieved, thereby disrupting the pH gradient. Thus, dynamic or covalent coatings are used to suppress the EOF and reduce the adsorption of proteins onto the capillary surface.

CIEF was introduced by Hjertén and coworkers in the mid-1980s [131-133] and has continued to develop through new innovations over the last two decades [134-140]. Although CIEF is known for its high resolution of peptides and proteins resulting in resolutions of up to 0.005 p*I*s units or less, it is limited because of the low solubility of proteins around their p*I*. Very high protein concentration within separation zones result in precipitation. In addition, CIEF can only be used for the separation of compounds that have an accessible p*I* (pH 3-10). Thus, CIEF is not a suitable technique for the analysis of pharmaceutical compounds.

1.6.2 Electric Field Gradient Focusing

Electric field gradient focusing (EFGF) is an equilibrium gradient technique based on counterbalancing the bulk flow of the solution and the electrophoretic velocity of an analyte

[126, 128, 141-143]. Separation of analytes occurs as a result of differences in their electrophoretic mobility, and they are focused at spatially distinct points along the separation channel. A non-uniform electric field gradient is created along the length of a separation channel by use of a series of electrodes and a semipermeable membrane. The bulk flow of the solution is adjusted such that the net mobility of an analyte across the channel is equal to zero and at this point the analyte is focused.

The separation of proteins using EFGF was initially demonstrated by Koegler and Ivory [141] using an electrochromatographic column where a linear electric field gradient was generated using a fluted cooling jacket. Charged proteins were focused and separated against a constant flow in the buffer in a packed dialysis tube. Although the technique successfully illustrated an alternative equilibrium gradient technique, the approach was slow and cumbersome. In 1996, Greenlee and Ivory [144] illustrated the focusing of proteins using an electric field gradient formed from an axial conductivity gradient. The apparatus was much simpler to construct and use than that developed by Koegler and Ivory [141]. Thereafter, Huang and Ivory developed an EFGF technique employing an array of electrodes whose voltages were monitored using a computer controlled circuit board. Separation and focusing of proteins was possible at concentrations greater than 50 mg/mL in a packed column format without protein precipitation.

The advantages of EFGF over CIEF are that a single continuous buffer is used and it is not limited to analytes with an accessible p*I*. However, in EFGF electrolysis chemical products at the buffer-electrode interface may be produced from the electric field gradients in the case where metal electrodes are used [145, 146]. The use of semipermeable membranes [126, 141] or a porous salt bridge structure [147] can eliminate problems associated with the

use of metal electrodes. However, their application is limited to large analytes that cannot penetrate the membrane or salt bridge.

1.6.3 Temperature Gradient Focusing

Temperature gradient focusing (TGF) was recently developed by Ross *et al*. [148] for the separation of ionic species in a microchannel or a capillary device. In addition to achieving a 10000-fold concentration enhancement, TGF has been shown to successfully separate a wide range of analytes including fluorescent dyes, amino acids, DNA, proteins, and particles. Analytes are focused by balancing the electrophoretic velocity of an analyte versus that of the bulk flow of solution in the presence of a temperature gradient. An appropriate buffer, whose ionic strength is temperature dependent, is used to achieve the temperature gradient created by heating one end of the separation column while cooling the other.

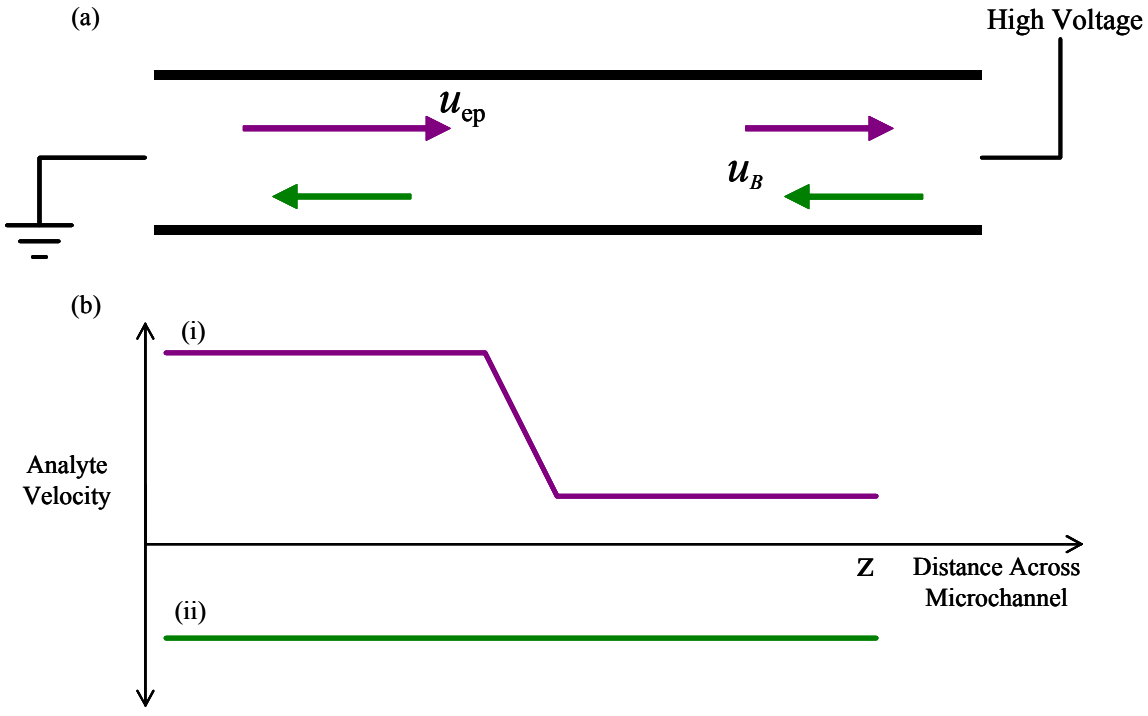

Figure 1.13 (a) Schematic diagram of focused analyte in a separation channel. (b) Velocity profile in presence of a temperature gradient of (i) the electrophoretic mobility of the analyte (ii) the bulk flow.

The apparent (net) velocity, μ_{app}, of an analyte is given by the summation of the electrophoretic mobility, μ_{ep}, and the bulk flow, μ_B. Both μ_{ep} and μ_B are in opposite directions and can be balanced in the presence of a velocity gradient such that μ_{app} is zero where the analyte is focused (Figure 1.13). It should be noted that the bulk flow in this case is created by a combination of an applied hydrodynamic pressure and the magnitude of the electroosmotic flow [148].

The main advantage of TGF is its simplicity of instrumentation that does not require the use of embedded electrodes or semipermeable membranes like EFGF. Although TGF is applicable to all charged analytes including proteins, it cannot be used for the separation of neutral analytes that do not posses an electrophoretic velocity. In addition, it is limited in the types of buffers used because they have to exhibit a temperature dependent ionic strength.

1.6.4 Micellar Affinity Gradient Focusing

Micellar affinity gradient focusing (MAGF) is an equilibrium gradient technique developed mainly for separation of neutral analytes [149]. It is a combination of two techniques, MEKC and TGF. Similar to MEKC, a pseudostationary phase is added to the mobile phase making the separation of neutral analytes possible, while a temperature gradient is applied to achieve focusing. However, MAGF does not require a temperature dependent buffer. On application of both voltage and hydrodynamic pressure, the pseudostationary phase and running buffer move in opposite directions. The micelles move from a region of high retention to low retention (right to left) and the running buffer moves in the opposite direction (left to right) as shown in Figure 1.14. In this case the micelles in the running buffer are negatively charged. In the region of low retention, the analyte interacts less with the micelles and therefore migrates in the direction of the running buffer. In the

region of high retention, the analyte interacts strongly with the micelles and thus moves in the direction of the micelle solution. In between the two regions, the net velocity of the analyte is zero and it is at this point the analyte focuses.

In MAGF a spatial gradient is created based on the retention factor of an analyte (k') in a pseudostationary phase . The net velocity of an analyte is given by equation 1.22,

$$u_T = (u_B + u_{ep})[1/(1+k')] + u_{MC}.[k'/(1+k')] \quad (1.22)$$

where u_B is the bulk flow velocity resulting from the magnitude of EOF and the hydrodynamic pressure applied, μ_{ep} is the electrophoretic velocity of the analyte, u_{MC} is the velocity of the micelles, and k' is the retention factor.

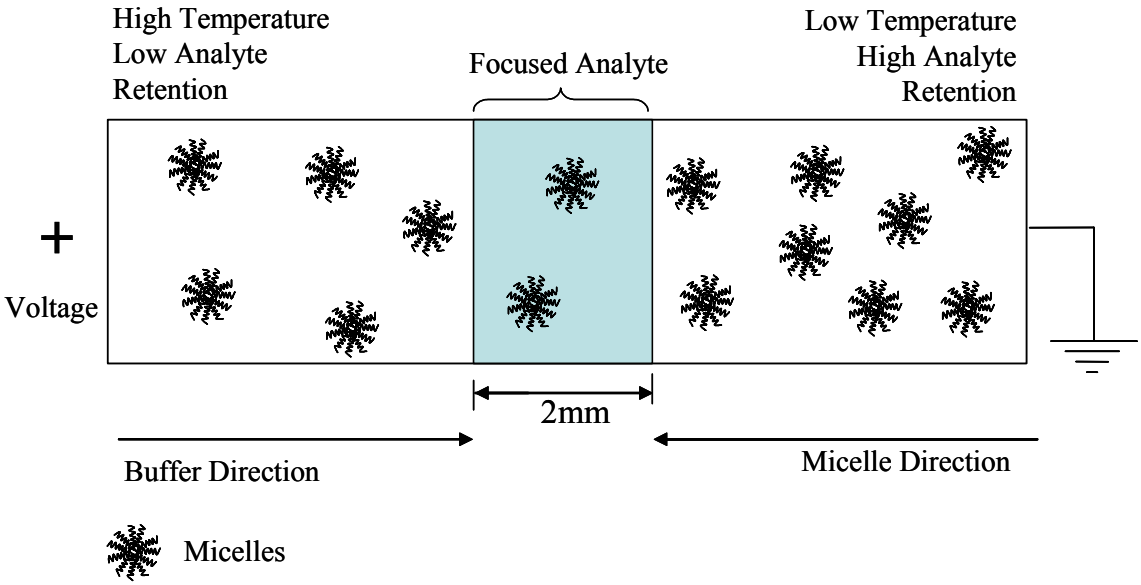

Figure 1.14 Schematic of MAGF microchannel with negatively charged micelles in the mobile phase. Modified from [149].

The retention factor k', is the ratio of the time the analyte spends in the pseudostationary phase to the time it spends in the mobile phase such that,

$$k' = K \cdot \beta \quad (1.23)$$

where K is the partition coefficient (equation 1.14) and β is the phase ratio (ratio of the volume of pseudostationary phase to the volume of mobile phase). In MAGF, a gradient in retention factor can be accomplished by either generating a gradient in the partition coefficient or the phase ratio, or a combination, of the two. Either one of these factors must be temperature dependent.

In the case where conventional micelles are used for MAGF, the retention gradient is a result of a gradient in both the partition coefficient and the phase ratio. The CMC, a temperature dependent parameter, increases with temperature, thereby decreasing the phase ratio at high temperatures. This causes a low concentration of micelles at the heated end and a high concentration at the cooled end of the separation channel. The analyte preferentially partitions in either the pseudostationary phase or the running buffer and it focuses at some point where its net mobility is zero. In the case where the pseudostationary phase is a polymeric surfactant, the phase ratio is constant and only the partition coefficient changes with temperature. As a result the temperature dependence is a result of the hydrophobic interactions between the analyte and the polymeric surfactant.

A schematic diagram of the apparatus used for both MAGF and TGF by Ross *et al*. [148-151] is shown in Figure 1.15. A microchannel, in the form of a capillary or a microchip device, is inserted between sample and waste reservoirs. A vertical translation stage connected to a tube is used to control the hydrodynamic pressure and to load the buffer into the microchannel. Two metal blocks, usually made of copper are, used to heat the separation channel on one end to 80 °C and cool the other end to 10 °C, thereby achieving a linear temperature gradient across the capillary. A fluorescence microscope, connected to a mercury arc lamp, is used for the detection of fluorescent analytes in the microchannel. High

voltage from an external power source is applied during the separation to generate the bulk flow.

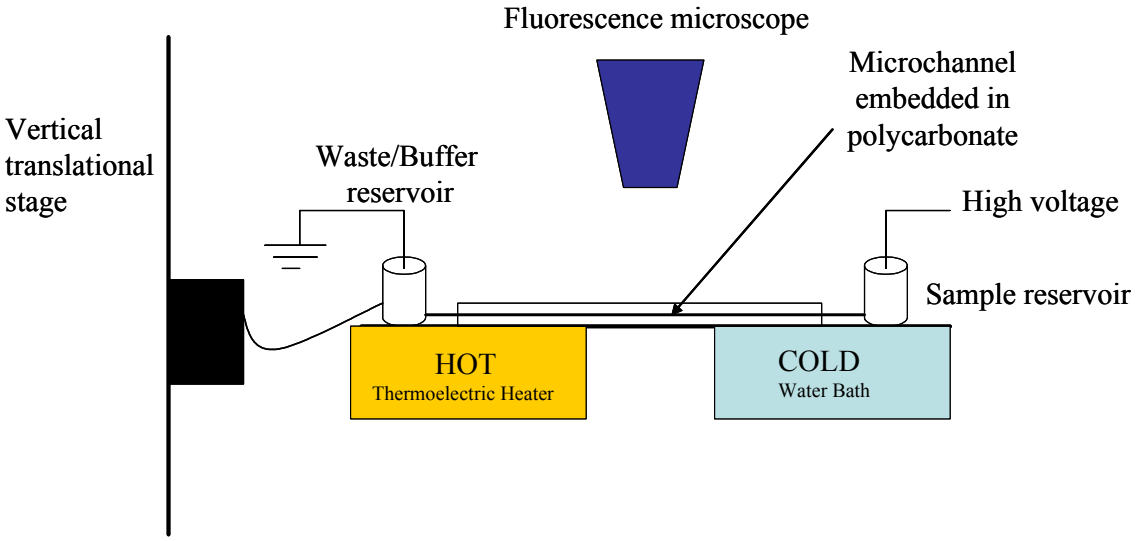

Figure 1.15 A schematic representation of MAGF/TGF apparatus. Modified from [148].

Part 3 Mass Spectrometry Coupled to Capillary Electrophoresis

1.7 Mass Spectrometry

Although CE is a favorable separation technique due to its high efficiency separations, short analysis time and low sample size consumption, it lacks qualitative analysis when UV detection is employed. In addition, UV detection is incapable of detecting trace levels of pharmaceuticals and their metabolites, and suffers from relatively low sensitivity. An ideal detector for CE should afford universal detection, sensitivity and selectivity without compromising separation efficiency [152]. The coupling of CE to mass spectrometry (MS) alleviates the aforementioned limitations. MS detection introduces a second dimension of separation in the gaseous phase by the analyte mass-to-charge (m/z) ratio in addition to separations based on the charge-to-size ratio provided by the CE in liquid phase [153].

MS is a tool primarily used to determine the molecular weight of an unknown compound and can also provide structural information. The main components of the instrument consist of (1) an ionization source, (2) a mass analyzer, and (3) the detector. A schematic representation of an electrospray-time of flight mass spectrometer (ESI TOF MS) is shown in Figure1.16.

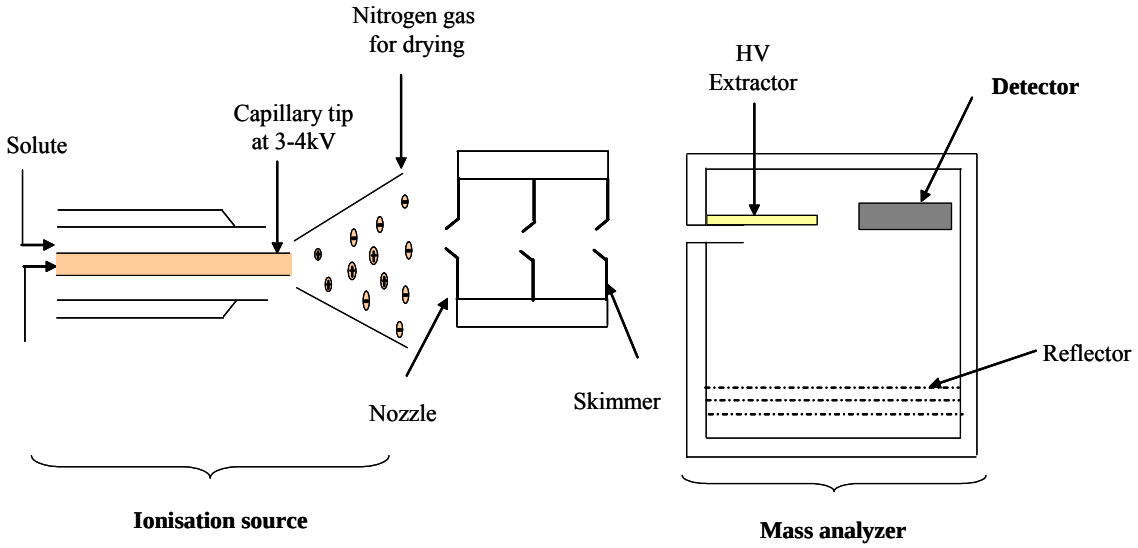

Figure 1.16 Schematic representation of the components of a time of flight mass spectrometer. Modified from [154].

In the ionization source, molecules can be converted into a gaseous phase and are ionized using different ionization techniques. Although, electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) are the most commonly used for biochemical analyses, ESI is suitable for CE/MS applications mainly because it performs well with liquid-based separation techniques such as CE. In addition, ESI is tolerant of different types of solvents, operates at wide solvent flow rates, and is capable of generating intact multiply-charged ions of delicate biochemical analytes [155]. In ESI, the analyte is dissolved in a polar solvent and pumped through a narrow stainless steel capillary at flow rates from 2 to 5 μL/min. Although the mechanism of gas phase ion formation is debatable

[156-160], three main processes are involved: droplet formation, droplet shrinkage, and gaseous ion formation. The electrostatic force (3 to 4 kV) exerted at the tip of the capillary causes the analyte solvent to merge in the shape of a "Taylor cone" [161]. Formation of charged droplets in the form of an aerosol results from the cone and the process is assisted by the presence of a nebulizing gas, usually nitrogen, flowing coaxially outside the capillary. The droplets are reduced in size due to solvent evaporation resulting from the warm flow of nitrogen gas in front of the ionization source. Figure 1.17 illustrates the basic principle of electrospray ionization in a typical ESI source. Factors such as the applied potential, solvent flow rate, solvent properties and capillary diameter influence the size of the initially formed droplets [155]. Eventually, ions free from solvent are released and directed into the skimmer that has a moderate vacuum after which they go into the mass analyzer. The mass analyzer is maintained at high vacuum to avoid ion collision with air molecules.

The main function of a mass analyzer is to separate ions from the ionization source based on their mass-to-charge (m/z) ratios. There are several types of mass analyzers including magnetic sectors, quadrupoles, fourier transform, and time-of-flight (TOF). However, for the purpose of this dissertation, the discussion here is based on the TOF mass analyzer. In a TOF mass spectrometer ions are separated based on their differences in velocity in a flight tube. Mass analysis is based on the principle that after acceleration of ion under a fixed kinetic energy, E, the velocity of the ions, v, is inversely related to the square root of their m/z values such that,

$$v = \left(\frac{2zV}{m} \right)^{1/2} \tag{1.24}$$

where z is the charge of the ion, V is the acceleration potential and the kinetic energy, E, is given by the product zV. At the same kinetic energy, lighter ions travel faster and reach the

detector faster than the heavier ones. The analyte flight time, t, over a given length in a flight tube, L, is calculated using equation 1.25.

$$t = \frac{L}{v} = L\left(\frac{m}{2zV}\right)^{1/2} \tag{1.25}$$

The time spectrum is converted into a mass spectrum by mass calibrating the instrument, where the flight times of two separate ions with known masses are measured. The mass spectrum consists of a plot of the relative intensity, or abundance, versus the m/z ratio.

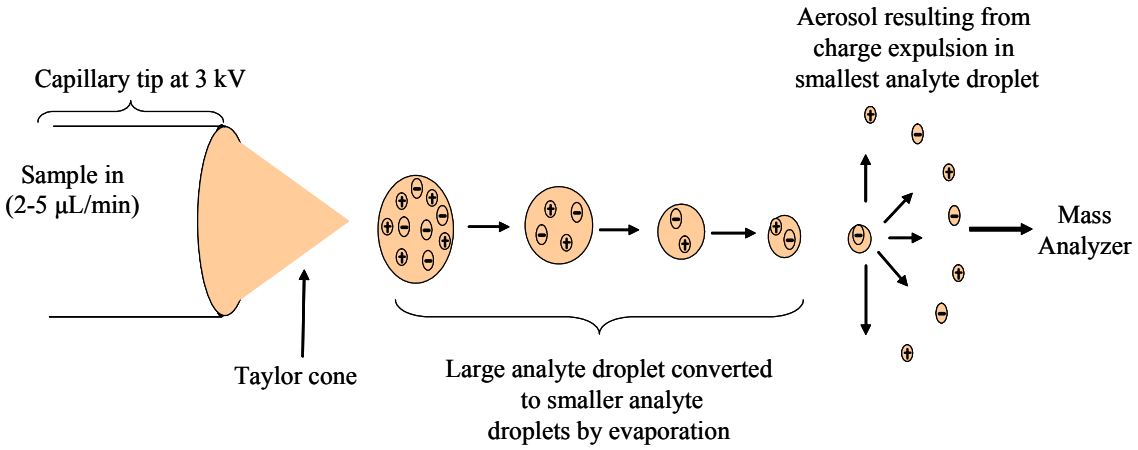

Figure 1.17 Formation of ions charged form a charged droplet in electrospray ionization.

1.7.1 Coupling of Mass Spectrometry to Capillary Electrophoresis

When coupling CE to MS as an online detector, the required electrical contact at the capillary outlet can be accomplished by use of a sheathflow or sheathless interface. In a sheathflow interface, a coaxial sheath liquid is introduced and serves as the electrical contact, while in a sheathless interface the electrical contact is provided by coating a conducting material applied at the tip of the separation column. The most commonly used conducting material is gold [162, 163]; however, other conducting materials such as carbon [164] and graphite [153, 165-169] have been used. Sheathflow interfaces are commonly used in commercial instruments [170] and offer several advantages including simple fabrication,

reliability, and easy implementation. In addition, the electrospray in a sheathflow interface is less dependent on the EOF and, thus, increases the range of separation conditions that can be used. However, the main limitation of the sheathflow interface is that the analyte concentration is often reduced by dilution with the sheath liquid leading to decreased sensitivity. On the other hand, in sheathless interfaces, the conductive coating material often has a short lifetime as the coating is susceptible to degradation. In addition, the coating procedure is often time consuming and requires special instrumentation.

The coupling of CEC and MEKC to MS is more suitable due to the wide separation applications offered by these techniques over CZE. This is because both CEC and MEKC techniques separate analytes based on their electrophoretic mobility as well as the partitioning of the analyte into a stationary or pseudostationary phase. Thus, the separation of difficult analytes, such as chiral molecules and neutrals, is possible [171]. However, the coupling of online MEKC-ESI MS presents a number of problems due to the non-volatility and high surface activity of conventional micelles [172]. In addition, a large background signal that interferes with analyte detection and a fouling of the ionization source is caused by micelle dissociation. This ultimately compromises the sensitivity of the mass spectrometer.

Relative to conventional micelles, polymeric surfactants can be used in MEKC-ESI MS separations to decrease the interference of the surfactant with the mass signal. In 2001, Shamsi [171] demonstrated the enantiomeric separation of BOH using the polymeric surfactant poly (sodium *N*-undecenyl-L-valinate), poly-L-SUV, using MEKC-ESI MS. Recently Akbay *et al*. [173] investigated the use of poly(sodium *N*-undecenoxy carbonyl-L-leucinate) for the simultaneous enantioseparation and detection of eight structurally similar

β-blockers with tandem UV and MS detection. In both cases, reasonable detection sensitivity was obtained; however, the use of polymeric surfactants can lead to reduced ionization efficiency resulting from the formation of an analyte-surfactant complex. To this date there are only three reports on the use of polymeric surfactants for MEKC-ESI MS separations [171, 173, 174].

Several alternative approaches have been investigated to eliminate the introduction of micelles or buffer additives into the MS such as the use of semi-permeable membranes [175] and partial-filling MEKC [176-179]. An alternative approach is reversing the direction of the charged micelles by adjusting pH [83] or polarity [180] such that the analytes and micelle move in opposite directions. Although such techniques have been demonstrated, they are only applicable to certain groups of analytes and are not as stable in preventing micelles entering the MS [176].

PEM coatings have recently been applied as a means to circumvent these problems and facilitate the coupling of CE to MS. Zhu *et al*. [181] successfully coupled a PEM-coated capillary to ESI MS for the analysis of β-blockers and benzodiazepines. The coating consisted of a bilayer of PDADMAC and the polymeric surfactant poly-SUS. Baseline separation was achieved for the four benzodiazepines using the PEM-coated capillary, while no separation was obtained using a bare capillary. In a recent study, the same group investigated the separation of labetalol diastereoisomers using a chiral PEM coating coupled to MS [96].

Katayama *et al*. [103] used cationic PEM coatings for the separation and MS detection of four cationic β-blockers. Efficient separations with reduced peak tailing were obtained as a result of the coulombic repulsion between the coating and analytes. The cationic PEM

coating was found suitable for CE/MS experiments that require the EOF to introduce analytes to the MS, as opposed to neutral coatings such as poly (vinyl alcohol) where the EOF is suppressed.

Bendahl *et al*. [108] investigated the use of a sheathless interface in CE/MS for the detection of alkaline compounds at low pH. In order to minimize poly (vinlylsulfonate), (PVS) background in the MS, they used a polybrene/PVS coated capillary. The use of MS detection gave comparable or better sensitivities than UV detection in their studies.

Tachibana *et al*. [182] recently used an SMIL coating on a quartz microchip interface to MS for the analysis of peptides and amino acids. The coating, which was similar to that used by Katayama *et al*. [102], consisted of polybrene and DS. An enhanced and stable EOF under low pH conditions was observed in the coated microchip, making sample injection possible. In addition, the electrophoretic mobilities of the amino acids increased under these conditions. In comparison to a coated capillary, an uncoated quartz microchip at low pH conditions exhibited a weak EOF making it difficult to control the flow in the microchannel.

1.8 Scope of Dissertation

This dissertation investigates the applications of polymeric surfactants for the separation of both chiral and achiral compounds such as phenols, benzodiazepines, binaphthyl derivates, β-blockers, and coumarin dyes that are often difficult to separate due to their properties. Both open-tubular capillary electrochromatography and micellar affinity gradient focusing techniques were developed in these studies.

In Chapter 2, OT-CEC is used for the separation of achiral phenols and achiral benzodiazepines. A PEM coating is constructed *in situ* by the alternate adsorption of negatively and positively charged polyelectrolytes. The polyelectrolytes consists of the

cationic poly (diallydimethylammonium chloride) (PDADMAC) and the anionic polymeric surfactant, poly (sodium undecenyl sulfate) (poly-SUS). The performance of the coating is evaluated by electrochromatographic experiments. The reproducibility of the PEM coating is evaluated by calculating the run-to-run and capillary-to-capillary relative standard deviation (RSD) of the EOF. In addition, the chromatographic performance of the coating using OT-CEC and MEKC is compared for the separation of benzodiazepines.

In Chapter 3, the use of a chiral PEM coating for chiral separation of three binaphthyl derivatives and two β-blockers is discussed. The PEM coating consists of the chiral poly-L-lysine hydrobromide (poly-L-lysine), and the chiral polymeric dipeptide surfactant, poly (sodium-undecenyl-L-leucine alanate) (poly-L-SULA). The application of chiral poly-L-lysine in the PEM coating, as an alternative to PDADMAC, leads to an increase in selectivity and resolution. A number of parameters that have an effect on the resolution and separation efficiency are optimized. In addition the effect of varying the amino acid order on the polymeric surfactant is investigated. The column performance is evaluated by calculating the run-to-run and capillary-to-capillary RSD of the EOF. Finally the coupling of this chiral OT-CEC column with mass spectrometry is investigated for the detection of labetalol diastereoisomers.

In Chapter 4, the coupling of a PEM coated capillary with ESI MS is investigated for the analysis of β-blockers and benzodiazepine analytes. The PEM coating consists of a single bilayer of PDADMAC and poly-SUS constructed by the alternate adsorption of these polyelectrolytes on a fused-silica capillary. Coupling a PEM coated capillary to MS is advantageous as it minimizes the introduction of polymeric surfactants into the MS. This

eliminates the detection interference caused by the nonvolatile micelle like pseudostationary phase. The effect of pH and applied voltage on the separation is investigated.

In Chapter 5, the application of a polymeric surfactant for the simultaneous concentration and separation in MAGF is described. The separation of three coumarin dyes that are neutral and hydrophobic is performed using the polymeric surfactant, poly-SUS. The use of poly-SUS compared to conventional micelles of SDS is advantageous as poly-SUS enables the focusing of coumarin dyes at a faster rate. A series of separations with varying focusing times and input concentrations are performed in order to asses the effect on peak intensity. Reproducibility is evaluated by computing the RSD of consecutive runs. In addition, the effect of varying the temperature gradient is investigated in the separation the of three coumarin dyes.

Finally, Chapter 6 concludes of the studies in this dissertation and highlights the ongoing studies to develop a hybrid technique of OT-CEC and MEKC.

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CHAPTER 2

ACHIRAL SEPARATIONS USING AN ACHIRAL POLYELECTROLYTE MULTILAYER COATING IN OPEN-TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY*

2.1 Introduction

In recent years, there has been a growing interest in the use of capillary electrochromatography (CEC) due to its high separation efficiency [1, 2] and its compatibility with mass spectrometry [3, 4]. This versatile technique provides a suitable alternative to capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE) and micellar electrokinetic capillary chromatography (MEKC). As mentioned in Chapter 1, CEC is a hybrid micro-column electroseparation technique that combines the selectivity of high-performance liquid chromatography (HPLC) and the efficiency of capillary electrophoresis (CE) [5-7]. High separation selectivity is achieved by combining the electrophoretic mobility and the partitioning coefficients between the stationary phase and the mobile phase of the analytes [8]. As an electrically driven approach, CEC yields a plug-like profile for analyte movement across the capillary with reduced dispersion resulting in high peak efficiency [9-11].

CEC encompasses different modes of operation, two of which are packed-column CEC (PC-CEC) and open-tubular CEC (OT-CEC) [8, 12]. In PC-CEC, the stationary phase is packed into the silica capillaries while in OT-CEC the stationary phase is coated onto the inner surface of the capillary. There are a number of disadvantages associated with PC-CEC which limit its practical application. First, the fabrication of stable frits that maintain an

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unrestricted flow and retain packed material is a major challenge in PC-CEC. Second, difficulties in achieving stable baselines, stable currents, and reproducible migration times arise due to the formation of air bubbles around the packing materials and the frits [10]. Thus, Pressurization at both ends of the column is required to prevent bubble formation inside the capillary [13]. Third, preparation methods of stationary phases used in PC-CEC are usually time consuming and complicated.

In OT-CEC the capillary coating may be described as permanent or dynamic depending on the attachment of the coating to the surface of the capillary wall [14]. Permanent coatings are achieved by derivatization of the silanol groups on the capillary wall followed by covalent bonding with a polymeric material [15]. Although covalently modified capillaries are very stable, they are laborious and time consuming to prepare [16, 17]. A dynamic coating is typically prepared by rinsing the capillary with a solution containing the coating agent or by adding a small amount of the coating agent to the mobile phase [18, 19]. Dynamic coatings are adsorbed to the capillary wall via electrostatic interactions and hydrogen bonding. While these interactions are weaker than covalent bonds, multiple electrostatic interactions ensure a stable coating. Decher et al. introduced a multilayer procedure that employs electrostatic interaction between oppositely charged macromolecules[20, 21]. In this procedure, thin films can be constructed on a layer-by-layer basis on a hydrophilic surface, by alternately exposing positive and negative polyelectrolytes on a substrate [22, 23]. Recently, simple coating procedures have been developed where the coating material is physically adsorbed to the capillary wall by flushing successive multiple ionic polymer layers (SMIL) across capillaries [24, 25].

Currently the use of OT-CEC is of interest because of its economical use of polymeric surfactants which are time consuming to synthesize [26, 27]. Traditionally polymeric surfactants have been used for separations in MEKC [28, 29] where the polymeric surfactant is added to the mobile phase. A drawback of this method is that a large amount of polymeric surfactant is consumed in the separation. Another advantage of OT-CEC is the possibility of coupling an OT-CEC separation with electrospray ionization mass spectrometry detection because there is little interference of the polymeric surfactant with the analyte of interest. In addition, the possibility of clogging the ionization source with polymeric surfactant is eliminated.

Our laboratory has recently investigated the use of a polyelectrolyte multilayer (PEM) coating consisting of the polymeric surfactant, poly (sodium *N*-undecenyl glycinate) and poly (diallyldimethylammonium chloride) (PDADMAC) in OT-CEC [30]. The 10-bilayer PEM coating yielded remarkable endurance and stability even at extreme pH values. However, the PEM coating procedure was time consuming. It should also be noted that, the thickness of the PEM coating reduces the inner diameter of the capillary wall, which may lead to frequent blockage of the capillary.

In this chapter, the use of the polymeric surfactant, poly (sodium undecenyl sulfate), poly-SUS, and poly (diallyldimethylammonium chloride), PDADMAC, in a single bilayer PEM coating for OT-CEC separations is investigated. To evaluate the performance of the PEM coating, phenol and benzodiazepine analytes were examined. In addition, the separation of benzodiazepines analytes using the polymeric surfactant PEM coating in OT-CEC format was compared to the chromatographic performance of an uncoated silica capillary and MEKC.

2.2 Experimental

2.2.1 Reagents and Chemicals

PDADMAC and poly-SUS were used as the PEM coating reagents and their structures are shown in Figures 2.1 and Figure 2.2 respectively. PDADMAC polymer (MW = 200,000-350,000) was obtained from Aldrich Chemical (Milwaukee, WI). The phenol analytes (3,5-dimethylphenol, 4-methylphenol, phenol, 4-fluorophenol, 4-chlorophenol, 3-chlorophenol, 3-bromophenol) and the benzodiazepine analytes (flunitrazepam, temazepam, nitrazepam, diazepam, oxazepam, clonazepam, lorazepam) were purchased from Sigma Chemical Company (St.Louis, MO). Sodium chloride (NaCl) and the buffer solutions composed of sodium phosphate dibasic (Na_2HPO_4) and sodium borate ($Na_2B_4O_7$) were purchased from Fischer Scientific (Fair Lawn, NJ).

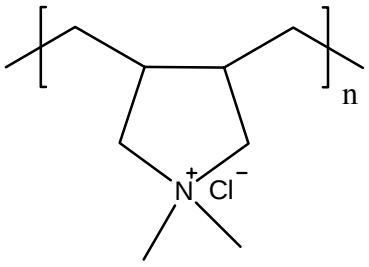


Figure 2.1 Structural representation of poly (diallyldimethylammonium chloride).

2.2.2 Synthesis of Poly (Sodium Undecenyl Sulfate)

The monomer of sodium undecenyl sulfate (mono-SUS) was synthesized in our laboratory from chlorosulfonic acid and 10-undecenyl alcohol, according to a previously reported procedure [31]. A schematic representation of the synthesis procedure is illustrated in Figure 2.2.

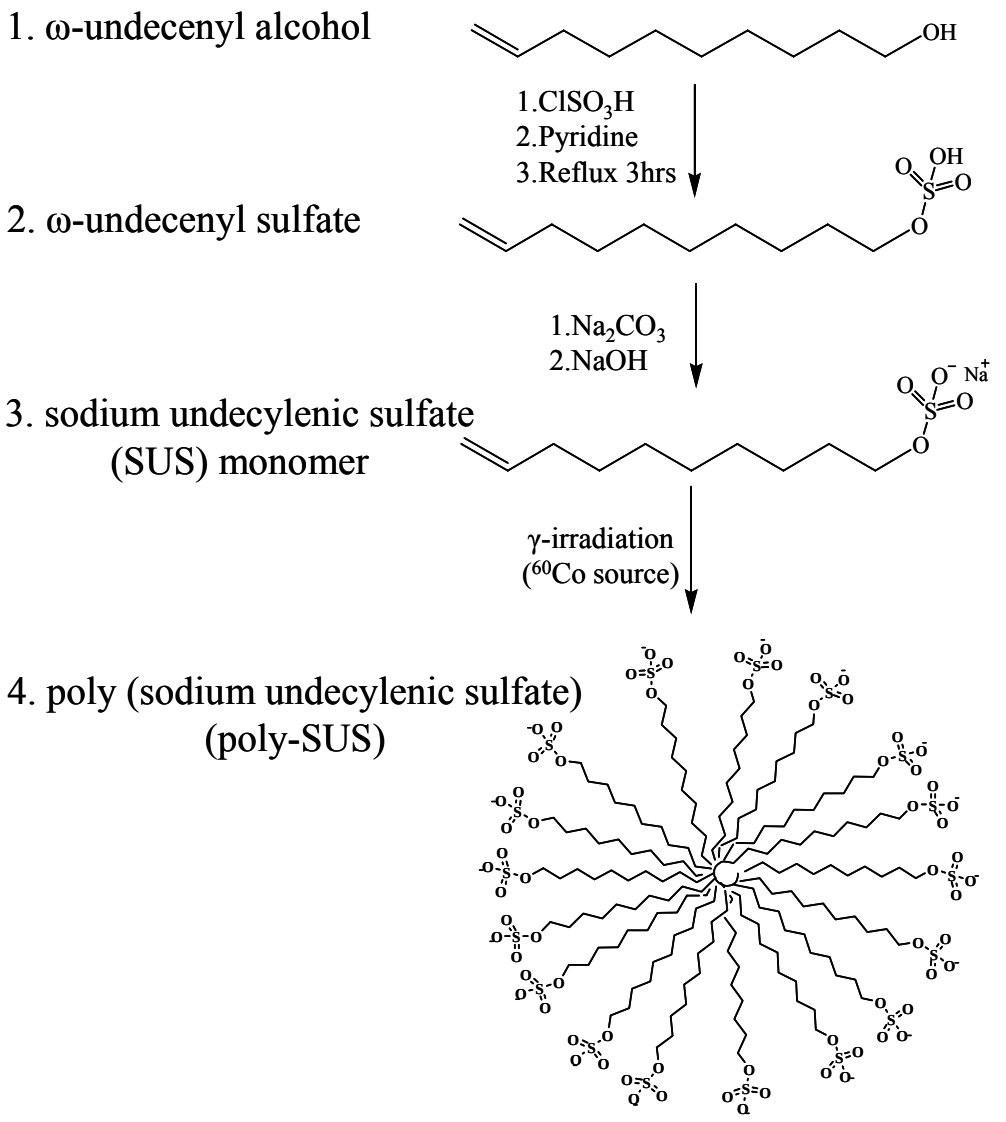


Figure 2.2 Synthesis scheme of poly (sodium undecenyl sulfate).

75 mL of chlorosulfonic acid was added dropwise to a round-bottomed flask containing 75 mL of pyridine and a magnetic stirrer. The round bottomed flask was placed in an ice bath because the reaction was exothermic and the resulting mixture was stirred vigorously. A solution containing 16.5 mL of ω-undecenyl alcohol and 75 mL of pyridine was slowly added to the above mixture. In order to obtain undecenyl sulfonic acid, the content of the resulting solution was refluxed with heat for 3 hours until a clear yellow solution was

formed. A heating mantle with a transformer set at 40 V was used to generate heat in the refluxing apparatus. A mixture of 4g of sodium hydroxide and 100 g of sodium carbonate dissolved in 600 mL of de-ionized water was added to undecenyl sulfonic acid solution and left to stir overnight. The product formed was a solution of sodium undecenyl sulfate (SUS) surfactant. Extraction of the aqueous phase of the SUS surfactant was performed 3 times by use of n-butanol in a separation funnel. The top organic layer in the separation funnel was placed in a rotary evaporator in order to obtain a dry product of the SUS surfactant. Thereafter the surfactant was purified by dissolving it in water and extracting with ethyl ether. The solution was then lyophilized resulting in a dry white powder. Recrystallization was performed by dissolving the product in heated isopropanol, filtering, cooling to room temperature and finally refrigerating. A vacuum dessicator was used to dry the crystals of the SUS monomeric surfactant overnight.

A 100 mM solution of the SUS monomeric surfactant was then polymerized by use of ^{60}Co γ radiation to form the poly-SUS. Thereafter, the solution was filtered under vacuum, dialyzed with a cellulose membrane with a 2000 Da molecular mass cutoff. The final poly-SUS solution was lyophilized to obtain the product form in the form of a powder. Elemental analysis calculations indicated a 97-99% purity of the product.

2.2.3 Instrumentation

All experiments were performed on an Hewlett-Packard ^{3D}CE capillary electrophoresis system (Hewlett-Packard, Walbronn, Germany) equipped with a diode array detector. The UV detector was set at 200 nm for phenol detection and 254 nm for benzodiazepine detection. Experimental data was collected and integrated using the HP Chemstation software. Fused-silica capillaries (58 cm total length, 50 cm effective length × 50 μm i.d.)

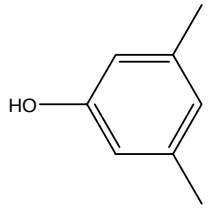
were purchased from Polymicro Technologies (Phoenix, AZ). Analytes were injected by pressure at 3 mbar for 3 s. The temperature of the capillary cassette was maintained at 20 °C and the applied voltage ranged from 15 kV to 20 kV. The electroosmotic flow (EOF), μ_{EOF}, was calculated using the equation

$$\mu_{EOF} = L_d L_t / V t_o \tag{2.1}$$

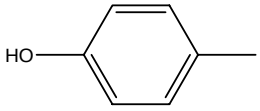
where L_d is the effective column length, L_t is the total capillary length, V is the applied voltage, t_o is the migration time of the EOF marker (methanol was used as the EOF marker). The migration time (t_o) of methanol was used in calculating the relative standard deviation (RSD) values in evaluation of reproducibility.

2.2.4 Buffer and Sample Preparation

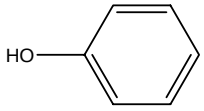
The analyte structures of the seven phenols and seven benzodiazepines used in this study are shown in Figures 2.3 and 2.4, respectively.



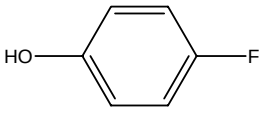
1. 3,5-Dimethylphenol



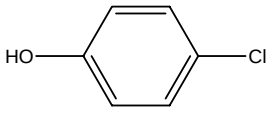
2. 4-Methylphenol



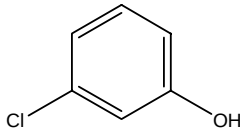
3. Phenol



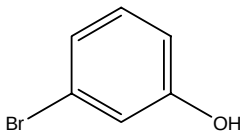
4. 4-Fluorophenol



5. 4-Chlorophenol



6. 3-Chlorophenol



7. 3-Bromophenol

Figure 2.3 Structural representation of phenol analytes investigated.

Standard stock solutions of the analytes were prepared in methanol at concentrations ranging from 0.1 to 0.5 mg/mL. The mobile phase consisted of a mixture of Na_2HPO_4 and $Na_2B_4O_7$ in the ratio of 1:1, buffered between pH 8.0 to pH 10.0. The pH was adjusted by titrating each buffer solution with either 1 M phosphoric acid (H_3PO_4) or 1 M sodium hydroxide (NaOH). Finally, the buffer solution was sonicated for 10 minutes and filtered through a 0.45 μm polypropylene nylon filter (Nalgene, Rochester, NY). The concentration of the $Na_2HPO_4/Na_2B_4O_7$ in mobile phase solution was varied from 15 mM to 50 mM.

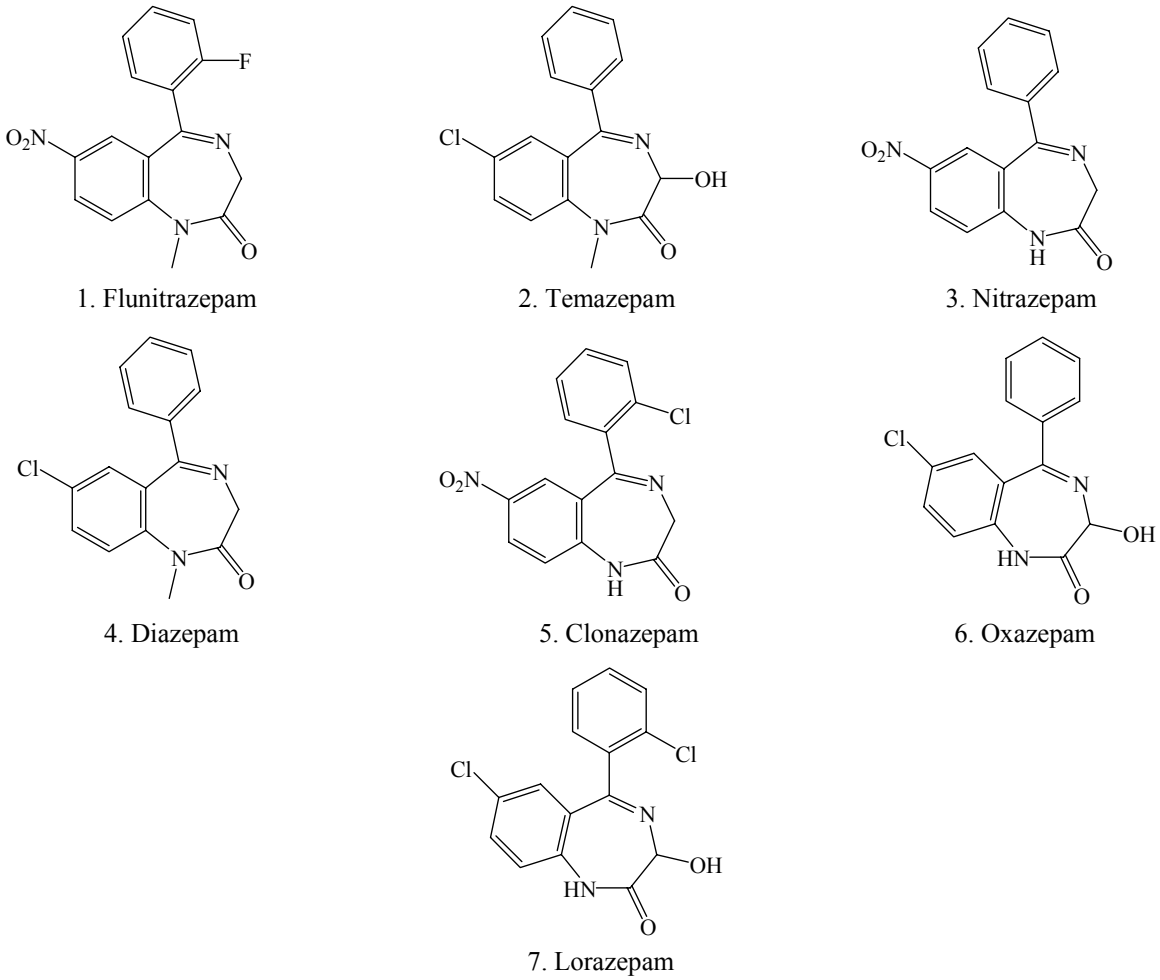


Figure 2.4 Structural representation of benzodiazepine analytes investigated.

2.2.5 Procedure for Polyelectrolyte Multilayer Coating

First, a detection window of 0.5 cm was prepared by burning off the external polyimide capillary coating and the polymer solutions were deposited on the inner capillary surface by using the flush function on the HP ^{3D}CE instrument. Initially the capillary was flushed with 1 M NaOH for 45 minutes and then with deionized water for 15 minutes. Next, the capillary was flushed with the 0.5% (w/v) PDADMAC in 0.2 M NaCl solution for 20 minutes followed by a 5 minute deionized water rinse. Finally, the capillary was flushed with 1% (w/v) poly-SUS for 20 minutes, and then with deionized water for 5 minutes. The total PEM coating procedure took less than 2.5 hours and the temperature of the cassette was maintained at 25 °C during this time. After coating, the capillary was conditioned with buffer until a stable baseline and current was achieved. The MEKC and uncoated fused-silica experiments were performed by first deprotonating the capillary with 1 M NaOH for 45 minutes and then conditioning with phosphate buffer for 20 minutes.

2.3 Results and Discussion

2.3.1 Reproducibility

Run-to-run and column-to-column capillary reproducibility is an important factor in evaluation of column performance. The RSD values of the EOF were obtained from five replicate analyses of the separation of seven phenol analytes. Figure 2.5 illustrates the electropherograms obtained from the separation of the phenol analytes on the first and fifth run. Table 2.1 reports the calculated RSD values for the migration times of the seven phenol analytes obtained by use of five consecutive runs.

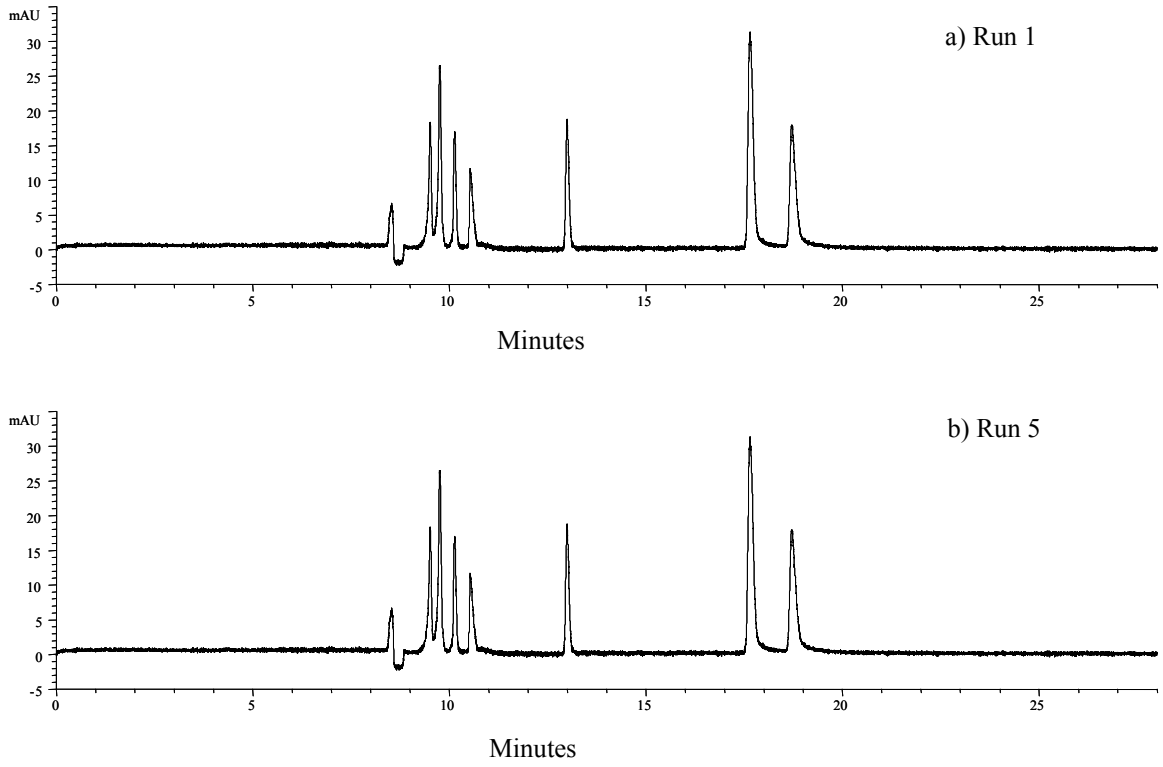


Figure 2.5 Run-to-run reproducibility of the separation of seven phenols.(a) Run 1 (b) Run 5. Conditions: PEM coating; 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; Mobile phase; 20 mM $Na_2HPO_4/Na_2B_4O_7$ at pH 9.2; temperature: 20 °C; injection: 3 s at a pressure of 30 mbar; applied voltage: 20 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection: 200 nm.

The EOF values from the first and the fifth runs were 2.932×10^{-3} $cm^2\ V^{-1}\ s^{-1}$ and 2.943×10^{-3} $cm^2\ V^{-1}\ s^{-1}$ respectively. These electropherograms also demonstrate the excellent run-to-run reproducibility with respect to the EOF and the migration times of each analyte. Table 2.2 reports the run-to-run and the column-to-column reproducibilities of the EOF in the separation of the phenol analytes that were found to be less than 1% in each case.

Table 2.1 Migration time reproducibilities of seven phenols analytes. The experiments were all performed on the same capillary. n = total number of runs. Conditions: same as for Figure 2.5.

Peak No.	Analyte	Average Migration Time t_m/min	%RSD (n=5)
1	3,5-dimethyl phenol	8.95	0.92
2	4-methyl phenol	9.19	0.92
3	phenol	9.50	0.95
4	4-fluorophenol	9.87	0.94
5	4-chlorophenol	12.08	0.52
6	3-chlorophenol	16.09	1.06
7	3-bromophenol	17.02	0.91

Table 2.2 Run-to-run and column-to-column reproducibilities of PEM capillary coating n = number of runs. Conditions: same as for Figure 2.5.

Capillary No.	EOF (average) t_m/min	Run-to-Run % RSD (n=3)	Column-to-Column[a] % RSD (n=4)
1	8.20	0.79	0.95
2	7.99	0.45	
3	7.93	0.08	
4	8.00	0.08	

[a] Column-to-column RSD values where computed from the average EOF values obtained from the four capillary columns.

2.3.2 Column Stability

Another important consideration of the PEM coating is the lifetime of the stationary phase. The endurance of the coating was tested in a PEM coated capillary by setting the

instrument to perform a series of runs over a period of five days. The mobile phase was replenished after every 20 runs in order to maintain current stability. All separations were performed at 20 °C using 20 mM phosphate/borate buffer (pH 9.2). The endurance of the coating was found to be more than 100 runs. After 120 runs there was a significant drift in EOF and in the migration times of the phenol analytes. This was potentially due to the detachment of the PEM coating from the capillary wall.

2.3.3 Separation of Phenols

OT-CEC has a number of parameters that can be varied in order to optimize the separation of particular analytes. One factor that influenced the separation of the phenols was the pH of the buffer. The pH changes the net charge of the analyte and hence the electrophoretic mobility of the analytes. Figure 2.6 is an illustration the separation of phenols at pH 8.0, pH 9.2 and pH 10.0 respectively. At the intermediate pH 8.0 a partial resolution of the analytes is observed (Figure 2.6a). At pH 9.2 (Figure 2.6b) a baseline separation is achieved. At pH 10.0 (Figure 2.6c), there was a significant increase in the EOF and, hence, an increase in migration time of the analytes. The change of the EOF with change in pH values indicates the dependency of EOF on the surface charge. The migration of phenol analytes in this case was influenced by the electrophoretic mobility of each analyte and the degree of interaction of the analyte with the PEM coating.

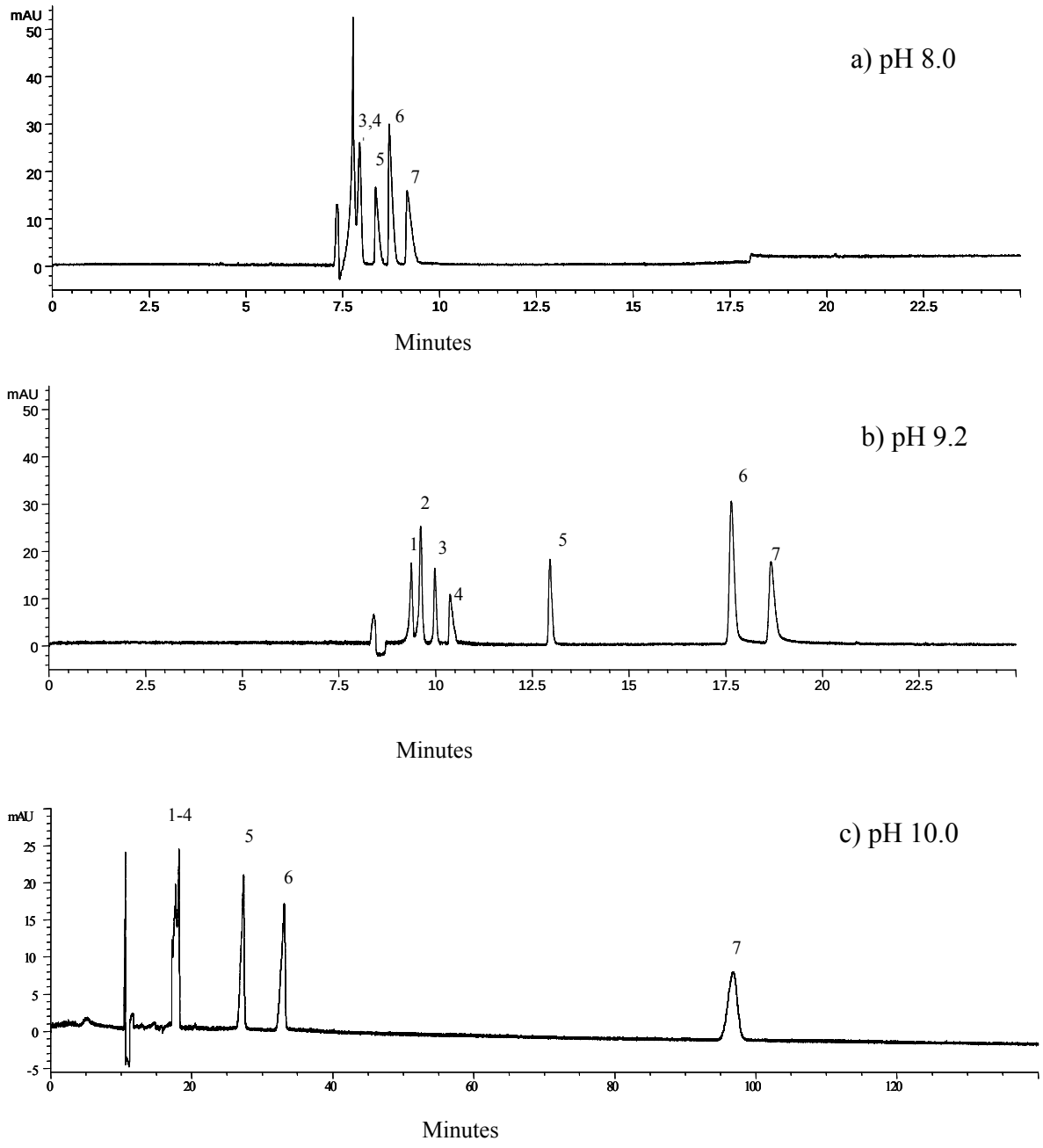


Figure 2.6 Effect of buffer pH on the OT-CEC on separation of phenols. Conditions: PEM coating: 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly (SUS); mobile phase: 20 mM $Na_2HPO_4/Na_2B_4O_7$, pH (8.0 to 10.0); temperature: 20 °C; injection: 3 s at a pressure of 3 mbar; applied voltage 20 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection: 200 nm

2.3.4 Pressure Studies

The PEM coating in OT-CEC column is analogous to the stationary phase of HPLC. To better understand the partitioning behavior of the analytes with the PEM coating, the separation of phenols in the coated capillary was investigated by use of pressure only. Thus, the separation mechanism in OT-CEC without an applied voltage was similar to that of HPLC. The phenol analytes were injected into the PEM coated capillary at various pressures and the applied voltage was zero. Figure 2.7 demonstrates the separation of seven phenols at varying pressures 2, 3, and 5 mbar, respectively. The partial resolution of seven phenols indicates that there was slight interaction between the analytes and the coating. As expected, the increase in pressure resulted in a decrease in the migration time and the resolution of the analytes. From the results it can be concluded that not only does electrophoretic mobility enhance the separation, but the hydrophobic interaction between analyte and the coating plays a role as well.

2.3.5 Separation of Benzodiazepines

The separation of benzodiazepines, a class of compounds that are used in clinical, pharmaceutical, and forensic studies, was also explored by use of the PEM coating. A baseline separation of the seven analytes was achieved in less than 30 minutes under optimized conditions (Figure 2.8). The separation of the benzodiazepine mixture using two different buffers, 30 mM borate/phosphate (pH 9.2) and 30 mM phosphate (pH 9.2), is shown in Figure 2.8. Both the resolution and elution time of the analytes changed by varying the buffer system. A slight tailing of benzodiazepine analytes was observed due to the presence of positively charged amine groups that are electrostatically attracted to the sulfonate groups on the coating.

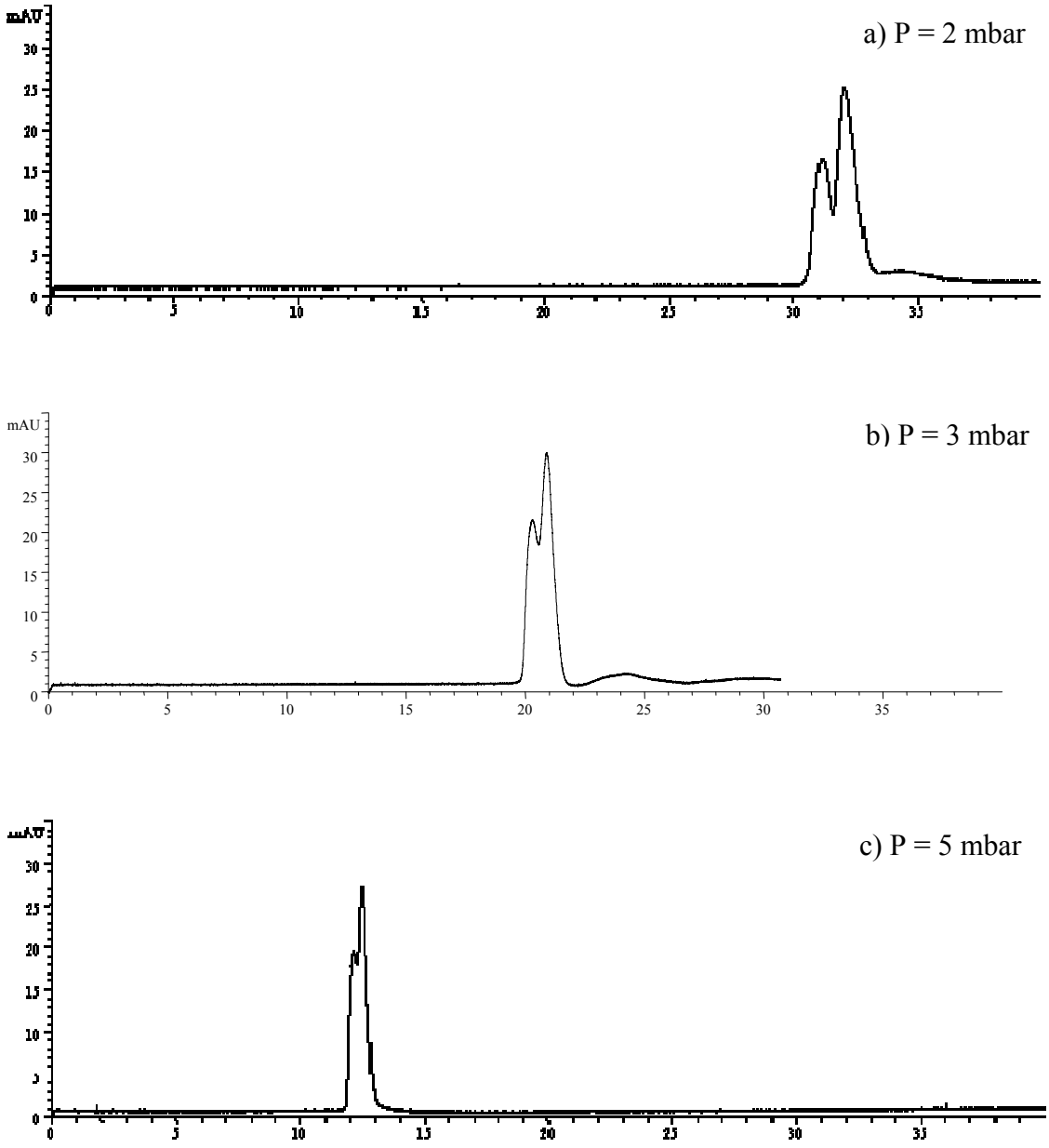


Figure 2.7 Effect of pressure in the separation of phenols. Conditions: PEM coating; 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; applied voltage: 0 kV; applied pressure: 2, 3 and 5 mbar respectively. Mobile phase: $Na_2HPO_4/Na_2B_4O_7$ (pH 9.2); temperature: 20 °C; injection size: 3 s at pressure of 30 mbar; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection: 200 nm.

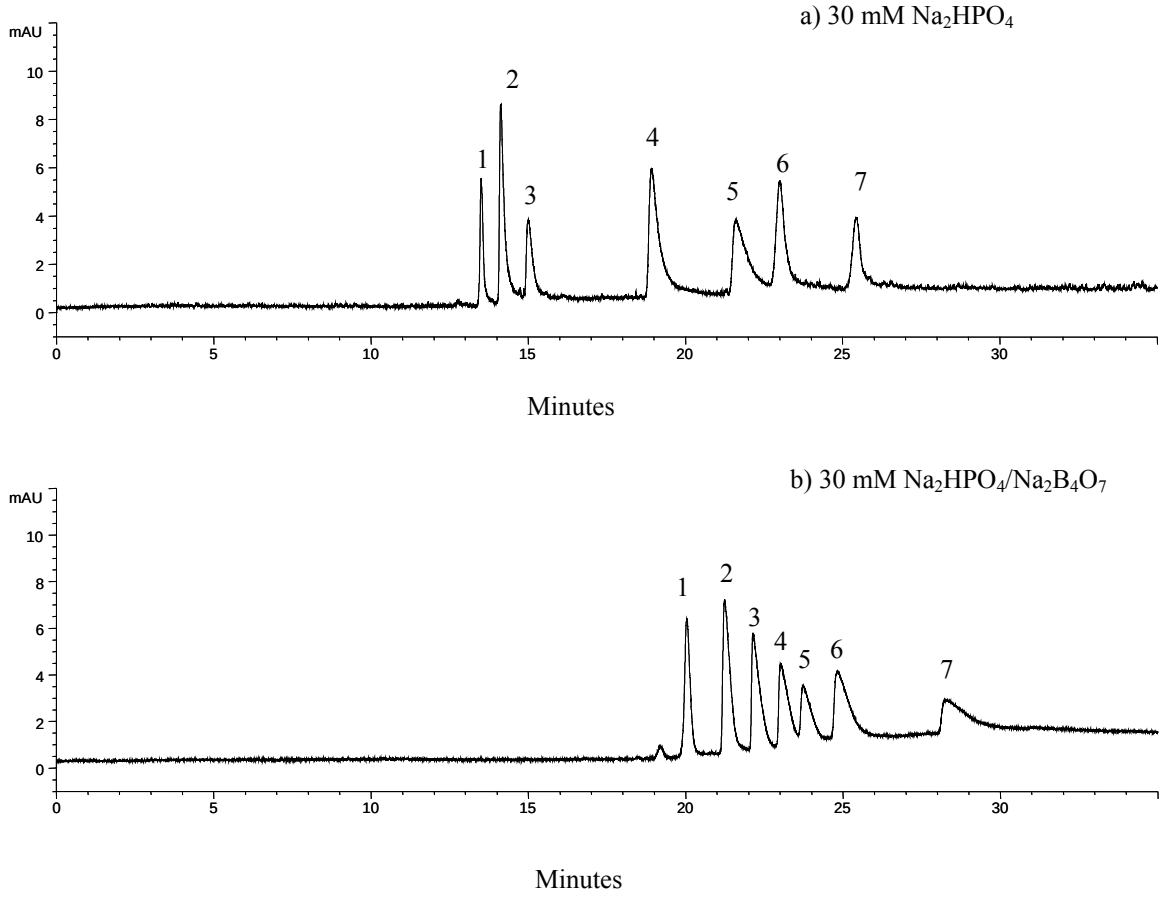


Figure 2.8 Separation of benzodiazepines using (a) 30 mM $Na_2HPO_4/Na_2B_4O_7$, pH 9.2 (b) 30 mM Na_2HPO_4, pH 9.2 Conditions: PEM coating: 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; temperature: 20 °C; injection size: 3 s at pressure of 3 mbar; applied voltage: 15 kV; capillary: 58 cm total length, 50 cm effective length, 50 µm i.d.; detection: 254 nm.

Better resolution and shorter elution times of the analytes were observed with phosphate buffer. In another study, the increase if borate/phosphate buffer concentration from 20 mM to 50mM was investigated for the separation of benzodiazepine analytes (Figure 2.9). An increase the buffer concentration from 20 mM to 30 mM resulted in baseline separation of the analytes. However, resolution between the analytes was compromised as the buffer concentration was increased to 50 mM.

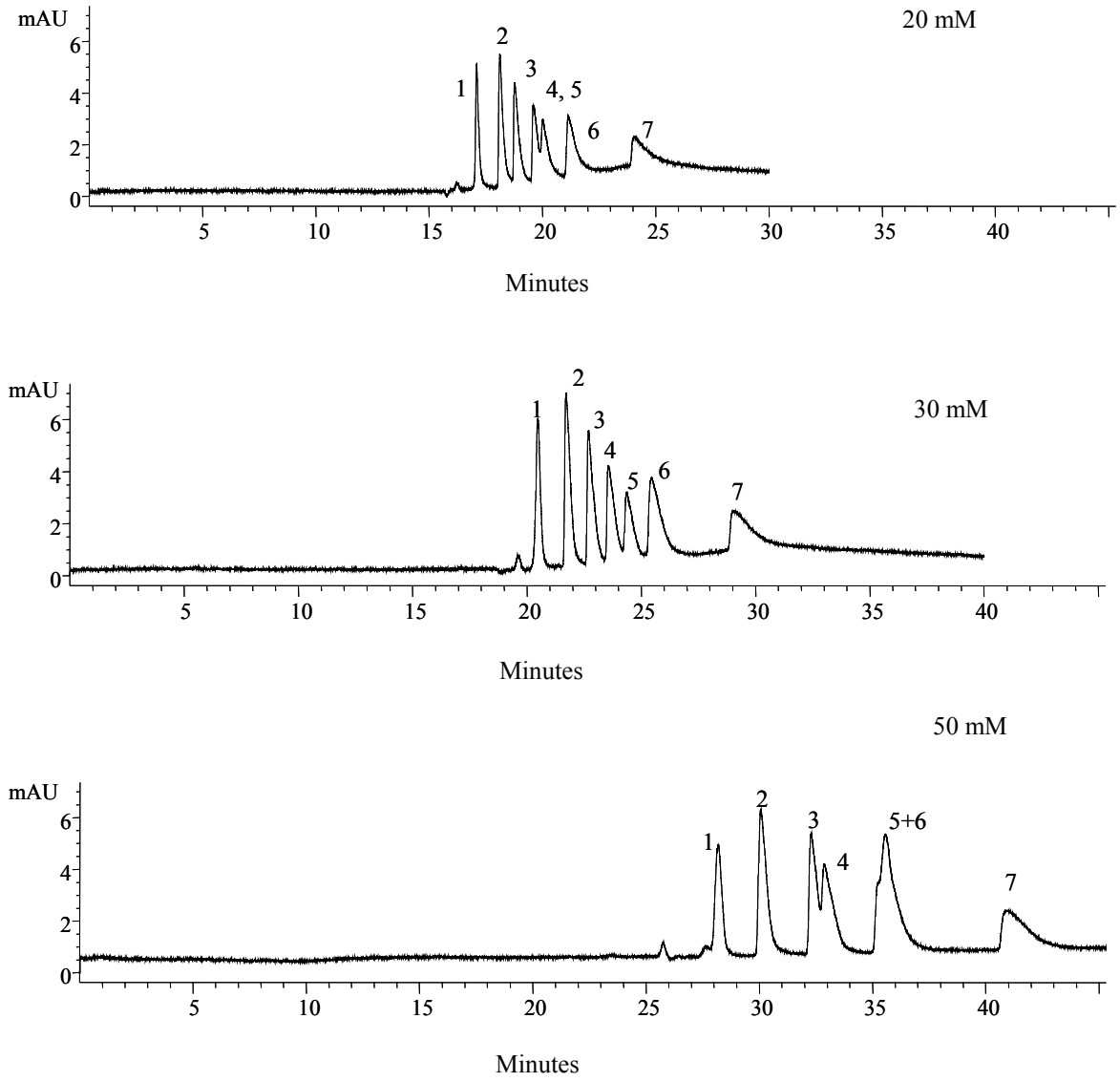


Figure 2.9 Effect of buffer concentration on the separation of Benzodiazepines. Mobile phase: $Na_2HPO_4/Na_2B_4O_7$ (pH 9.2); PEM coating: 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; temperature: 20 °C; injection size: 3 s at pressure of 3 mbar; applied voltage: 15 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection: 254 nm.

2.3.6 Comparison between Uncoated Silica and PEM Coated Capillaries

Previous reports have illustrated the usefulness of OT-CEC capillaries in the separation of some analytes. The significance of the PEM coating in optimizing a particular separation

was demonstrated by comparing separations on a uncoated silica capillary and a coated capillary. First, the importance of the PEM coating was tested using the benzodiazepine analytes. Figure 2.10a illustrates the separation of the benzodiazepines on an uncoated silica capillary where no separation of the benzodiazepines was achieved. In comparison, separation of seven benzodiazepines was observed when a PEM coated capillary was used in Figure 2.10b. The PEM coating acts as a stationary phase and the hydrophobic interaction between the hydrophobic polymer core and the non-polar moiety of each analyte enhanced separation. Separation of phenols on uncoated and PEM coated capillaries were also performed to investigate the role of the PEM coating as illustrated in Figure 2.11. Elution time of the phenol analytes was shorter in the uncoated capillary however, only six out of the seven phenols analytes were resolved. In the coated capillary baseline separation of the seven phenols was obtained at a longer elution time of 18 minutes. These results demonstrate the utility of the PEM coating to improve the resolution of these select analytes.

2.3.7 Separations of Benzodiazepines Using MEKC and OT-CEC

A comparative study of the separation of benzodiazepine analytes in MEKC and OT-CEC was performed. Figure 2.12 illustrates the separation of benzodiazepines using MEKC. In this study 0.1% w/v poly-SUS was added to the mobile phase. All other separation conditions were similar to those used in OT-CEC (Figure 2.8a). In Figure 2.8a, OT-CEC resulted in a baseline separation of the seven benzodiazepines and a shorter elution of all seven benzodiazepines. In MEKC, flunitrazepam and nitrazepam (peaks 1 and 3) coelute and longer retention times were observed. The elution order of the analytes changed when MEKC experiments were performed and this may imply a change in the separation mechanism. The separation mechanism for OT-CEC and MEKC is based on the electrophoretic mobility of

the analytes and hydrophobic interactions with the stationary phases. However, in MEKC the analytes partition into the pseudostationary phase while in OT-CEC analytes partition in the stationary phase coating.

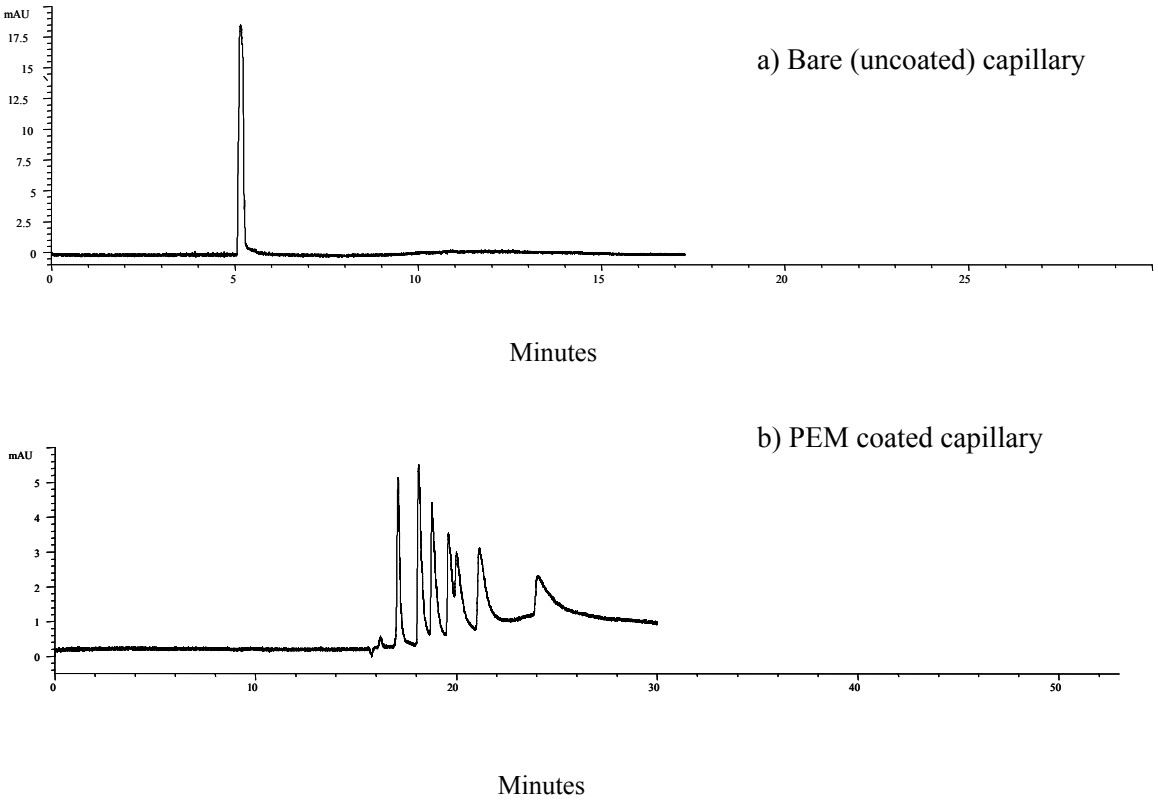


Figure 2.10 Separation of benzodiazepines a) Uncoated fused-silica capillary b) PDADMAC/ poly-SUS PEM coated capillary. Conditions: Mobile phase: 20 mM $Na_2HPO_4/Na_2B_4O_7$ (pH 9.2); temperature: 20 °C; injection: 3 s at a pressure of 3 mbar; applied voltage: 20 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection 254 nm.

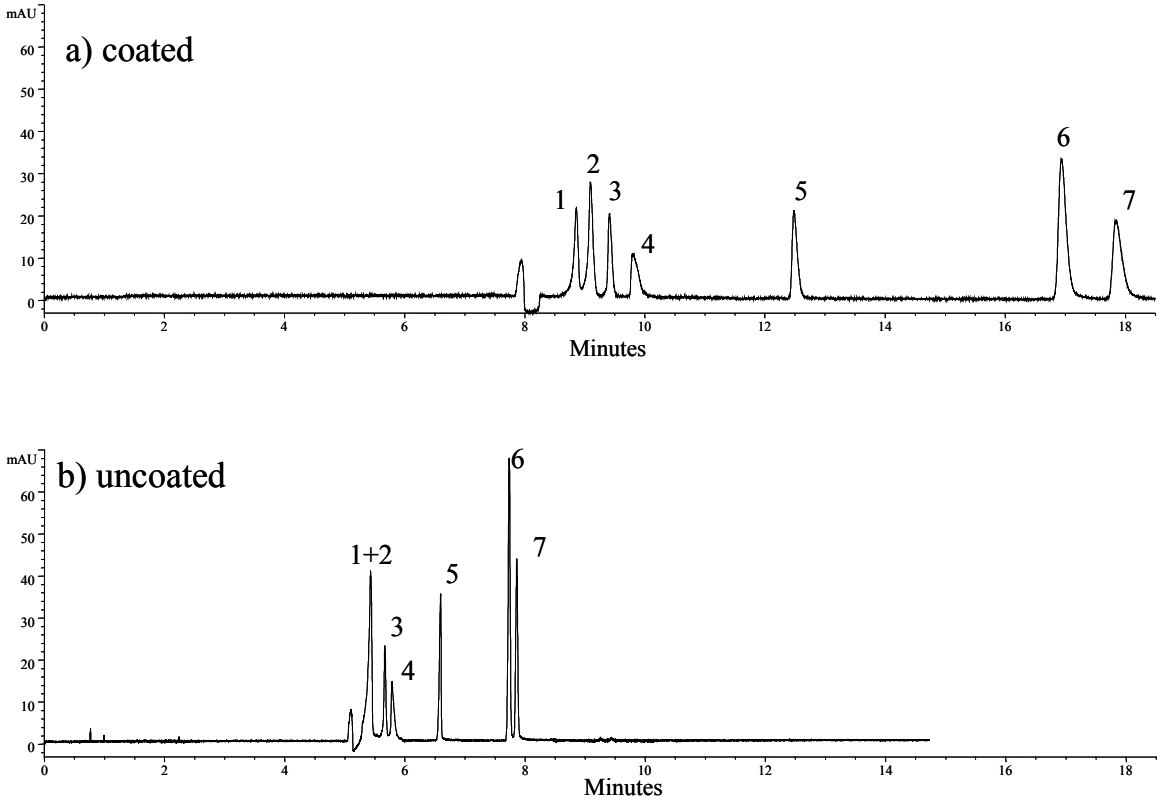


Figure 2.11 Separation of phenols (a) PDADMAC/ poly-SUS PEM coated capillary (b) Uncoated fused-silica capillary. Conditions: Mobile phase: 20 mM $Na_2HPO_4/Na_2B_4O_7$ (pH 9.2); temperature: 20 °C; injection: 3 s at a pressure of 3 mbar; applied voltage: 20 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection 254 nm.

2.4 Conclusions

The use of PEM coated capillaries has been shown to be a viable approach to OT-CEC. The PEM coating procedure is simple and takes a relatively short time (less than 2.5 hours). The PEM coating showed good selectivity towards achiral phenol and benzodiazepine analytes. The PEM coating exhibited excellent reproducibility with RSD values of less than 1.5%. The stability of the PEM coating has been shown to more than 100 runs. The coating has demonstrated superiority over MEKC and uncoated capillaries in the separation of benzodiazepines.

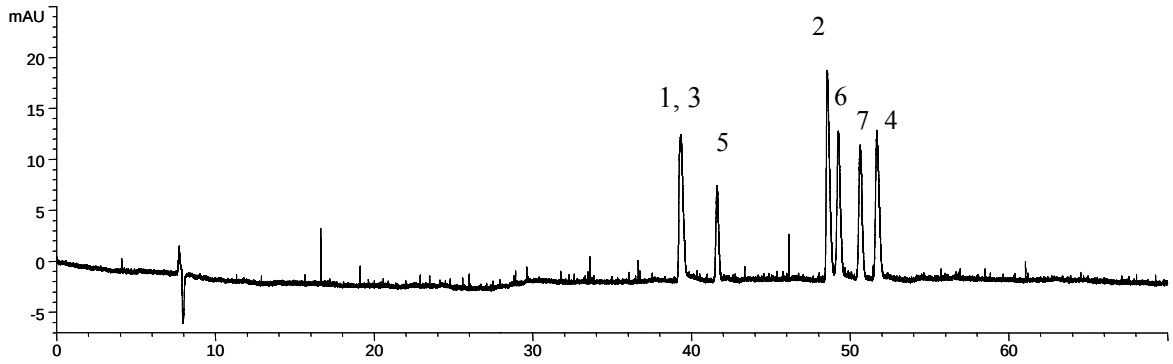


Figure 2.12 Separation of benzodiazepines using MEKC. Conditions: Mobile phase: 1% (w/v) poly-SUS in 20 mM Na_2HPO_4. Other conditions are same as Figure 2.8a.

2.5 References

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CHAPTER 3

CHIRAL SEPARATIONS USING A CHIRAL POLYELECTROLYTE MULTILAYER COATING IN OPEN-TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY*

3.1 Introduction

The enantioselectivity of chiral compounds is of great importance to the pharmaceutical industry. This is because a large number of pharmaceutical drugs exhibit chirality and as a result, one enantiomeric form may exhibit a desired physiological effect and the other enantiomeric form may be toxic [1]. For this reason, the Food and Drug Administration requires the separation of a drug into its individual enantiomers and the examination of its biological and toxicological effects before the drug can be commercialized [2]. Thus, the development of suitable methods for the separation of the pure enantiomers of a drug compound is important.

A number of separation techniques have been employed for the resolution of chiral drug compounds. These include high performance liquid chromatography [3], gas chromatography [4], supercritical fluid chromatography [5, 6], and capillary electrophoresis (CE) [7]. CE has emerged as a popular technique for the separation of enantiomers because of its high efficiency, as well as versatile applications in micellar electrokinetic chromatography (MEKC) [8] and capillary electrochromatography (CEC) [9].

Chiral separations may be performed using a pseudostationary phase that consists of chiral selectors such as cyclodextrins [10, 11], antibiotics [12], crown ethers [13], linear polymers [14], and micelles [15]. Polymeric surfactants have also been used for the chiral separation of charged and neutral enantiomers in MEKC [16-19]. In analytical separations,

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polymeric surfactants are preferred over conventional micelles due to their stability. Conventional micelles exhibit a limited stability due to the presence of a dynamic equilibrium between individual surfactant molecules and the micelle. Thus, organic solvents cannot be used in the background electrolyte (BGE) as they interfere with micelle formation. Unlike polymeric surfactants, conventional micelles require high surfactant concentrations above the critical micelle concentration in order to provide efficient separations.

Polymeric dipeptide surfactants have been traditionally employed for chiral separations in MEKC [20-25]. They provide a higher selectivity than the single amino acid polymeric surfactants due to the possession of two chiral centers. Shamsi et al.[20] were the first to report the use of a polymeric dipeptide surfactant. The authors observed a higher peak efficiency, resolution, and faster elution, when the polymeric dipeptide surfactant poly (sodium undecylenyl-L-leucyl valinate), poly-L-SULV, was used. This was compared to the chromatographic performance of alprenolol, propanolol, and 1,1′-bi-2-naphthyl-2,2′-dihydrogen phosphate, when poly (sodium undecylenyl-L-valinate), (poly-L-SUV), was used. Billiot et al.[22, 26-28] examined several factors that govern enantiomeric recognition in polymeric dipeptide surfactants. In their studies, Shamsi et al.[24] demonstrated that poly-L-SULV was a broad chiral selector that was capable of resolving various molecular classes of compounds. Although poly-L-SULV was shown to be a versatile chiral discriminator, poly (sodium undecylenyl-L-leucylalinate), poly-L-SULA, demonstrated a higher selectivity and a higher resolution for the separation of binaphthyl derivatives [29].

An alternative approach to MEKC is the use of OT-CEC, which was first introduced by Mayer and Schurig [30]. They achieved chiral separation of 1,1′-bi-2-naphthyl-2,2′-dihydrogen phosphate and 1-phenylethanol by use of capillaries coated with immobilized

Chiralsil-Dex. Katayama et al.[31, 32] introduced a simple coating procedure that utilized the PEM coating approach for the performance of achiral separations in OT-CEC. In general, a PEM coating is formed by alternately exposing a cationic and an anionic polyelectrolyte on a hydrophilic surface [33, 34]. The mechanism of formation of PEMs occurs *via* the ion exchange process that results in a stable coating [35-37]. Recently, our laboratory has investigated the use of PEM coatings for the separation of a number of chiral and achiral analytes indicating remarkable stability and reproducibility [38-40]. The advantage associated with use of PEM coatings is that they prevent fouling the ionization source when coupled with mass spectrometry (MS) [41]. In addition, excellent reproducibility and remarkable stability is achieved. Although this technique has a low phase ratio, selectivity can be enhanced by increasing the number of bilayers and the salt concentration added to the polymer deposition solutions [40].

In analytical separations, the most commonly used cationic polyelectrolytes for the construction of PEMs have been poly (diallydimethylammonium chloride), PDADMAC, [38-40, 42, 43] or polybrene [31, 32, 44]. The use of polypeptides in OT-CEC for the construction of PEMs is new; however, it is an important step for the chiral recognition of enantiomers as these systems mimic the biomembranes in the human body. Rmaile *et al*. [45] recently reported the use of optically active polypeptides consisting of poly-L-lysine and poly (glutamic acid) for chiral recognition of ascorbic acid in OT-CEC. Their work illustrated remarkable permeability and chiral selectivity.

In this chapter, the use of a PEM coating that consists of the polypeptide poly-L-lysine hydrobromide (poly-L-lysine) and the polymeric dipeptide surfactant poly (sodium undecylenyl-L-leucyl alanate) (poly-L-SULA) or poly (sodium undecylenyl-L-alanine

leucinate) (poly-L-SUAL) for the chiral separation of three binaphthyl derivatives and two β-blockers is investigated. Several experimental parameters are varied in order to optimize the separation conditions. The coating exhibits remarkable reproducibility and stability, and it endures over 290 runs. In addition, the coupling of chiral OT-CEC to MS by the use of the PEM-coated capillary is reported for the first time.

3.2 Experimental

3.2.1 Reagents and Chemicals

Poly-L-lysine hydrobromide (M_W = 150,000-300,000) and poly (diallydimethylammonium chloride) (M_W =150,000-300,000) were obtained from Sigma (St. Louis, MO). The chemical structures of poly-L-lysine and PDADMAC are shown in Figure 3.1 and Figure 2.1 (chapter 2) respectively. The pure enantiomers and the racemic mixtures of binaphthyl derivatives 1,1′-bi-2-naphthyl-2,2′-dihydrogen phosphate (BNP), 1,1′-bi-2-naphthol (BOH) and 1,1′-binapthyl-2,2′-diamine (BNA), as well as labetalol and sotalol were purchased from Sigma (St. Louis, MO). The chemical structures of these analytes are shown in Figure 3.2.

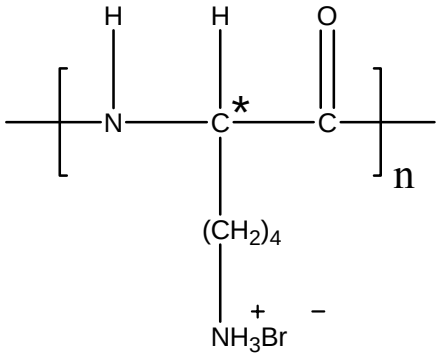
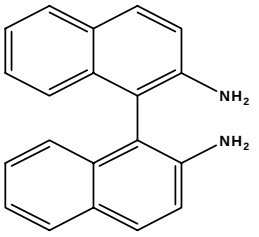
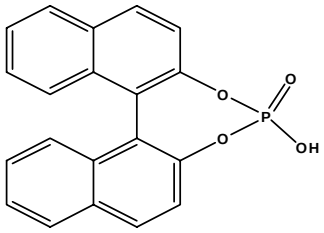


Figure 3.1 Chemical structure of poly-L-lysine hydrobromide. (* represents a chiral center).



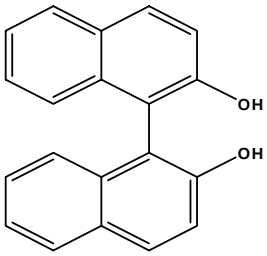
1,1'-binaphthyl-2,2'-diamine

(BNA)



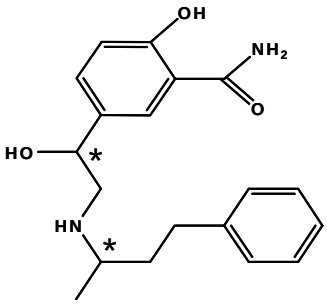
1,1'-binaphthyl-2,2'-dihydrogenphosphate

(BNP)

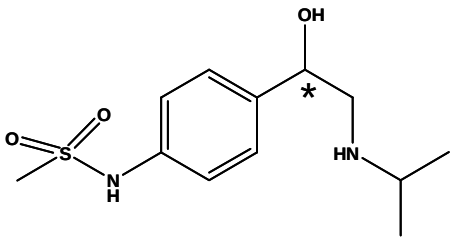


1,1'-bi-2-naphthol

(BOH)



Labetalol

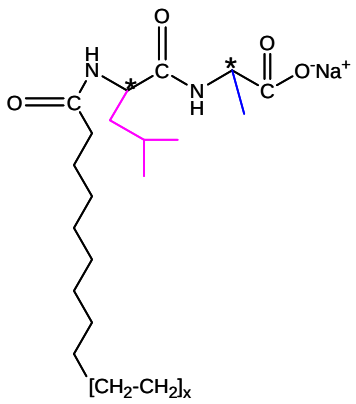


Sotalol

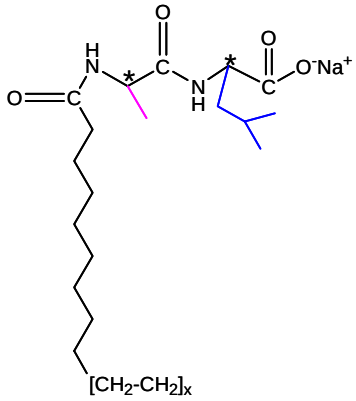
Figure 3.2 Chemical structures of analytes investigated. (* represents a chiral center).

Sodium borate ($Na_2B_4O_7$), tris(hydroxymethyl)aminomethane (Tris), hexylamine ($C_6H_{15}N$), methanol (MeOH), cyclohexylaminopropanesulfonate (CAPS), and sodium chloride (NaCl) were obtained from Fisher Scientific (Fair Lawn, NJ). Ultra pure grade ammonium acetate was purchased from Amresco Inc. (Solon, OH). Chemicals used for the synthesis of the polymeric dipeptide surfactant poly-L-SULA and poly-L-SUAL, (*N, N*-

dicyclohexylcarbodiimide (DCC), undecylenic acid, and *N*-hydroxysuccinimide (NHS)) were purchased from Sigma (St. Louis, MO). The dipeptides L-leucine-alanate and L-alanine-leucinate were purchased from BaChem Bioscience Inc (King of Prussia, PA). All chemicals were used as received.



poly (sodium N-undecylenyl- L-leucyl-alanate)
(poly-L-SULA)



poly (sodium N-undecylenyl- L-alanyl-leucinate)
(poly-L-SUAL)

Figure 3.3 Chemical structures of polymeric surfactants investigated.

3.2.2 Syntheses of Poly-L-SULA and Poly-L-SUAL

The surfactant monomers of sodium undecylenyl leucyl alanate (mono-SULA) and sodium undecylenyl alanine leucinate (mono-SUAL) were synthesized from the *N*-hydroxysuccinimide ester of undecylenic acid with the respective dipeptide according to the procedure reported by Wang and Warner [19]. The synthetic scheme of a typical monomeric dipeptide surfactant such as mono-SULA, is shown in Figure 3.4. The synthesis of a typical polymeric dipeptide involves three main steps. The first step involves the sythesis of *N*-hydroxysuccimide (NHS) ester of the undecylenic acid (Figure 3.4a), while the second, step

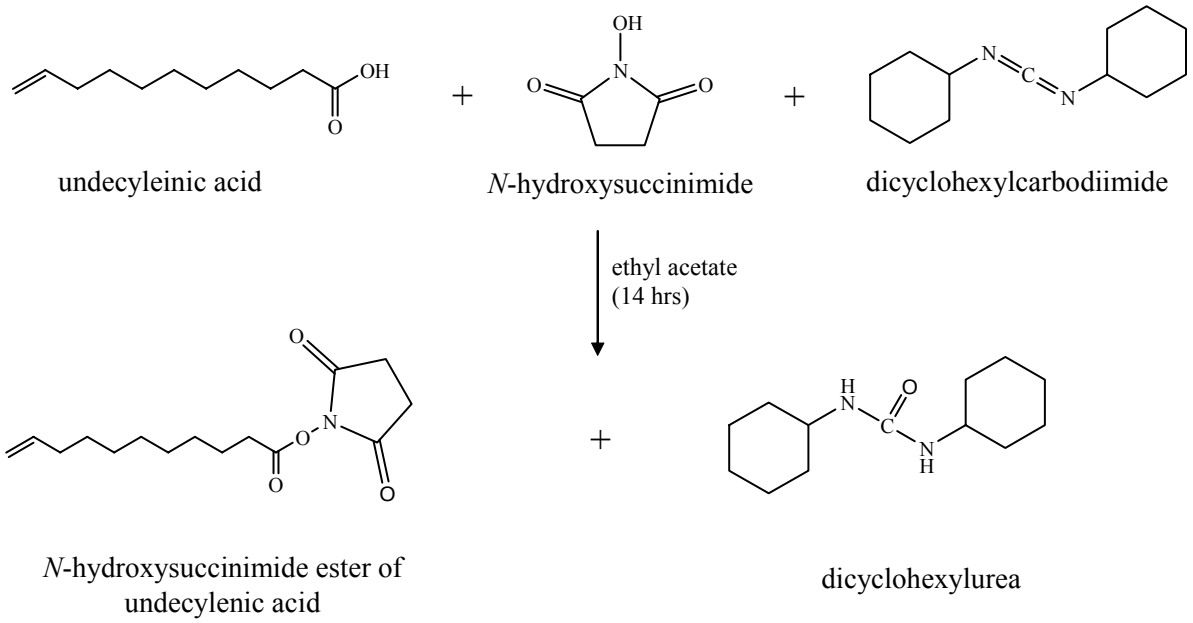
involves synthesis of the monomeric dipeptide surfactant synthesized (Figure 3.4 b). The third step involves the polymerization of the monomeric dipeptide surfactant.

To synthesize the NHS ester of the undecylenic acid, 25 g of NHS was dissolved in 500 mL of ethyl acetate in a round bottomed flask. 40 g of undecylenic acid dissolved in 170 mL of ethyl acetate was then added to the *N*- hydroxysuccinimide solution and allowed to stir overnight. The white precipitate of dicyclohexylurea formed was filtered and the resulting filterate was rotary evaporated to form a cloudy oily product. The NHS ester of the undecylenic acid was formed by recrystallizing by use of hot isopropyl alcohol. The NHS ester was dried under vacuum (Figure 3.4a).

20 mM of L-SULA monomer was sythesized by first dissolving 1.68 g of $NaHCO_3$ in 200 mL of triply distilled deionized water in a 2000 mL round bottomed flask. 2.34 g of the dipeptide, L-SULA, was then added to the mixture and stirred. A mixture of 5g of NHS of the undecylenic acid in 200 mL of THF was then added into the round bottomed flask and the resulting solution was allowed to stir for 16 hours. Thereafter, the solution was rotary evaporated at 40 °C to remove THF and a cloudy solution resulted. The cloudy solution was filtered several times by use of a buchner funnel and a glass filter under vacuum until a colorless filtrate was obtained.

The round bottomed flask containing the filtrate was then placed into an ice bath and allowed to chill for 2 hours after which 1 M HCl was added dropwise to adjust the pH to 1. The resulting white residue was washed with triply distilled deionized water and dried under vacuum. 2.52 g of $NaHCO_3$ dissolved 10 mL of triply distilled deionized water was then added to the white residue and stirred overnight. Thereafter, the solution was filtered again, chilled, and the pH was dropped by adding 1 M HCl.

(a) **Step 1**



(b) **Step 2**

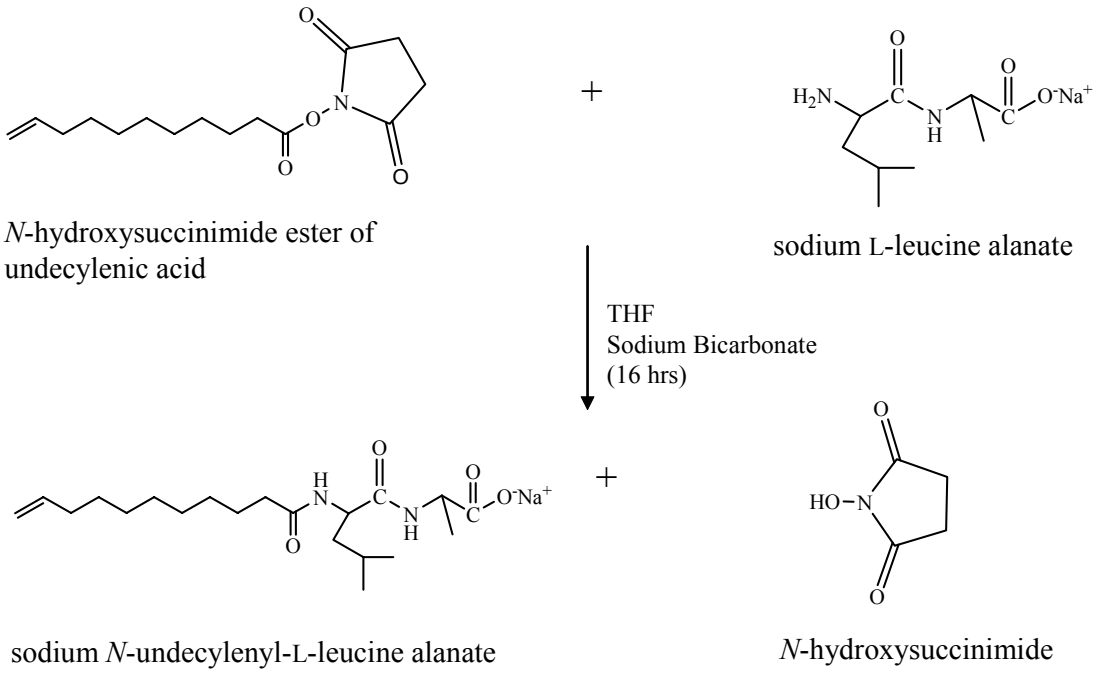


Figure 3.4 Synthetic scheme of (a) *N*-hydroxysuccimide ester (b) sodium *N*-undecylenyl-L-leucyl alanate.

The resulting acid product was washed several times with deionized water and dried under vacuum. An equimolar amount of $NaHCO_3$ was dissolved in deionized water was added to the L-SULA monomer and left to stir overnight. Finally, the monomer L-SULA monomer was filtered and lyophilized.

The CMC of L-SULA was determined by use of a surface tensiometer from CSC Scientific Company, Inc. (Fairfax, VA). 100 mM of L-SULA monomer was polymerized using ^{60}Co γ-ray source (70 krad/h) for 168 hours. Thereafter, a 2000 Da molecular weight cutoff filter was used to dialyze the aqueous solution of poly-L-SULA. The solution was then dried by used of a lyophilizer and ^{1}H NMR was used to confirm the form of the polymeric surfactant.

3.2.3 Instrumentation

3.2.3.1 OT-CEC

OT-CEC experiments were performed using an Hewlett-Packard ^{3D}CE system (Walbronn, Germany). The instrument was equipped with a diode array detector (DAD) for UV detection. In this study, the DAD was set at 220 nm for the detection of all analytes. The instrument also consisted of a 0-30 kV high-voltage built-in power supply, and Hewlett-Packard CE Chemstation software was used for control and data acquisition. Fused-silica capillaries employed in all experiments (50 i.d., 320 μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ). The effective length of the capillaries was 50 cm, while the total length was 57 cm.

3.2.3.2 OT-CEC/ESI MS

An Hewlett-Packard ^{3D}CE instrument (Palo Alto, CA) coupled to an electrospray ionization time-of-flight mass spectrometer (ESI TOF-MS) (Framingham, MA) was

employed for the chiral OT-CEC/ESI-MS experiments. All experiments were performed at room temperature. The sheath flow OT-CEC/ESI-MS interface was constructed by use of a CE-ESI sprayer from Agilent Technologies (Palo Alto, CA). The sprayer was capable of providing both a coaxial sheath liquid and a nebulization gas to assist the electrospray process. The capillary tip was set at an angle of 45 degrees relative to the direction of the ESI TOF-MS nozzle in order to obtain an optimum signal. The capillary tip was extended 0.5 mm from the sprayer tip. For all OT-CEC/ESI-MS experiments, the length of the poly-L-SULA-coated capillary was 61 cm. The sheath liquid was delivered at a flow rate of 2 □L/min. The ESI voltage applied on the sprayer was set at 2.85 kV in the positive mode. Data acquisition was performed in the range of m/z 100-1000 at a scan rate of 3 s per spectrum. The nozzle voltage was set at 150 V, and the skimmer voltage was set at 12 V. The nebulizer and curtain gases were both nitrogen, and the flow rates were optimized at 0.5 L/min and 0.7 L/min, respectively.

3.2.4 Buffer and Sample Preparation

The BGE for the OT-CEC experiments consisted of 100 mM Tris and 10 mM $Na_2B_4O_7$ at pH 10.2 or 300 mM CAPS and 50 mM $Na_2B_4O_7$ and 0.15% v/v hexylamine at pH 8.5. The pH of both BGEs was adjusted using 1 M sodium hydroxide (NaOH) or 1 M phosphoric acid (H_3PO_4). The BGE for the OT-CEC/ESI-MS experiments consisted of 25 mM ammonium acetate, and the pH was adjusted with ammonium hydroxide (NH_4OH) solution. All BGE solutions were filtered through 0.45 μm polypropylene nylon syringe filters, and then sonicated for 15 min to ensure proper degassing before use. The BGE was used to rinse the capillary for 2 min between runs. Analyte solutions were dissolved in either 50:50 or 80:20 methanol/water in order to give a final concentration of 0.1 mg/ml.

3.3 Procedure for Polyelectrolyte Multilayer Coating

All coating procedures were performed using the rinse function on the Hewlett-Packard ^{3D}CE system. Polymer deposition solutions consisted of 0.02% (w/v) poly-L-lysine with NaCl concentration ranging from 0 to 0.5 M and either 0.25-0.5% (w/v) poly-L-SULA or 0.25% (w/v) poly-L-SUAL with 0 to 0.5 M NaCl. First, the capillary was conditioned with 1 M NaOH for 30 min, followed by deionized water for 15 min. Next, the capillary was flushed with poly-L-lysine for the deposition of the first cationic layer. A 5-min rinse with de-ionized water was followed. Finally, the anionic polymeric dipeptide surfactant (poly-L-SULA or poly-L-SULA) was flushed for 5 min followed by a 5 min rinse with de-ionized water. The cationic and anionic layers constituted a single (one) bilayer. Consecutive bilayers were constructed by alternate 5-min rinses of poly-L-lysine and polymeric dipeptide surfactant in order to obtain the desired number of bilayers. After each polyelectrolyte deposition, the capillary was rinsed for 5 min with de-ionized water.

For the OT-CEC/ESI-MS experiments the coating protocol was similar to the one described above. However, a 61-cm long capillary was prepared using a 4-bilayer coating that consisted of 0.02% (w/v) poly-L-lysine and 0.25% (w/v) poly-L-SULA.

3.4 Calculations

Resolution (R_s), and selectivity (α), were calculated using the following equations,

$$R_s = \frac{2\left(t_{r2} - t_{r1}\right)}{w_1 + w_2} \tag{3.1}$$

$$\alpha = \frac{t_{r2} - t_o}{t_{r1} - t_o} \tag{3.2}$$

where t_o and t_r are the respective migration times of the neutral marker (MeOH) and the enantiomer respectively.

3.5 Results and Discussion

The optimization of several parameters in order to achieve baseline chiral separations was necessary. In this study, experimental parameters such as voltage, temperature, number of bilayers, salt concentration in the polymer deposition solutions, and type of chiral selector were investigated.

3.5.1 Enantiomeric Separation of Binaphthyl Derivatives

The three binaphthyl derivatives, BNP, BOH, and BNA are referred to as atropisomers because they possess a chiral plane of symmetry rather than an asymmetric carbon. The separation of these analytes using the PEM coating is illustrated in Figure 3.5. BNP, which is anionic at the given experimental conditions, eluted at a shorter time and gave the highest resolution of the 3 binaphthyl derivatives (Figure 3.5a). A possible explanation for this observation is that BNP is the least hydrophobic and, as such it does not penetrate deeply into the hydrophobic core of the polymeric dipeptide surfactant [28]. Figure 3.5b illustrates the separation of BOH under similar conditions as Figure 3.5a. BOH, which has a pKa value of 9.5, is partially anionic under the given experimental conditions. The reduction in peak efficiency, as compared to that of BNP, may be attributed to the higher hydrophobicity of BOH. The separation of BNA (Figure 3.5c) required the use of the organic modifier, methanol because it is the most hydrophobic and is neutral under the experimental conditions. In this case, the organic modifier solvates the solute, thus reducing hydrophobic interactions with the polymeric surfactant.

A comparative study was performed to investigate the separation of BNP using PDADMAC (Figure 3.6a) or poly-L-lysine (Figure 3.6b) as the cationic polymers employed for the construction of the PEM coating. As illustrated in Figure 3.6b, a one-bilayer coating, which was constructed by using poly-L-lysine as the cationic polymer, was sufficient to give a baseline separation of BNP in less than ten minutes. On the other hand, no resolution was obtained when PDADMAC was used, and the elution time of BNP was more than doubled. It is possible that poly-L-lysine enhances chiral selectivity because it is chiral while PDADMAC is achiral. In addition, the elution of the hydrophobic analyte BNA was not achieved when PDADMAC was used as the cationic polyelectrolyte. Based on the results PDADMAC forms a more hydrophobic PEM coating, which in turn, increases the elution time of the analytes.

3.5.2 Effect of Temperature

The influence of temperature on the separation of BNP enantiomers was investigated in Figure 3.7. In this study, the temperature was varied from 15 °C to 45 °C. As expected, a decrease in temperature resulted in an increase in retention time. This is probably due to a decrease in electrolyte viscosity upon increasing the temperature. The resolution of BNP gradually increased with a decrease in temperature. The optimal temperature for this separation was shown to be 15 °C since it yielded the best resolution and the minimal Joule heating.

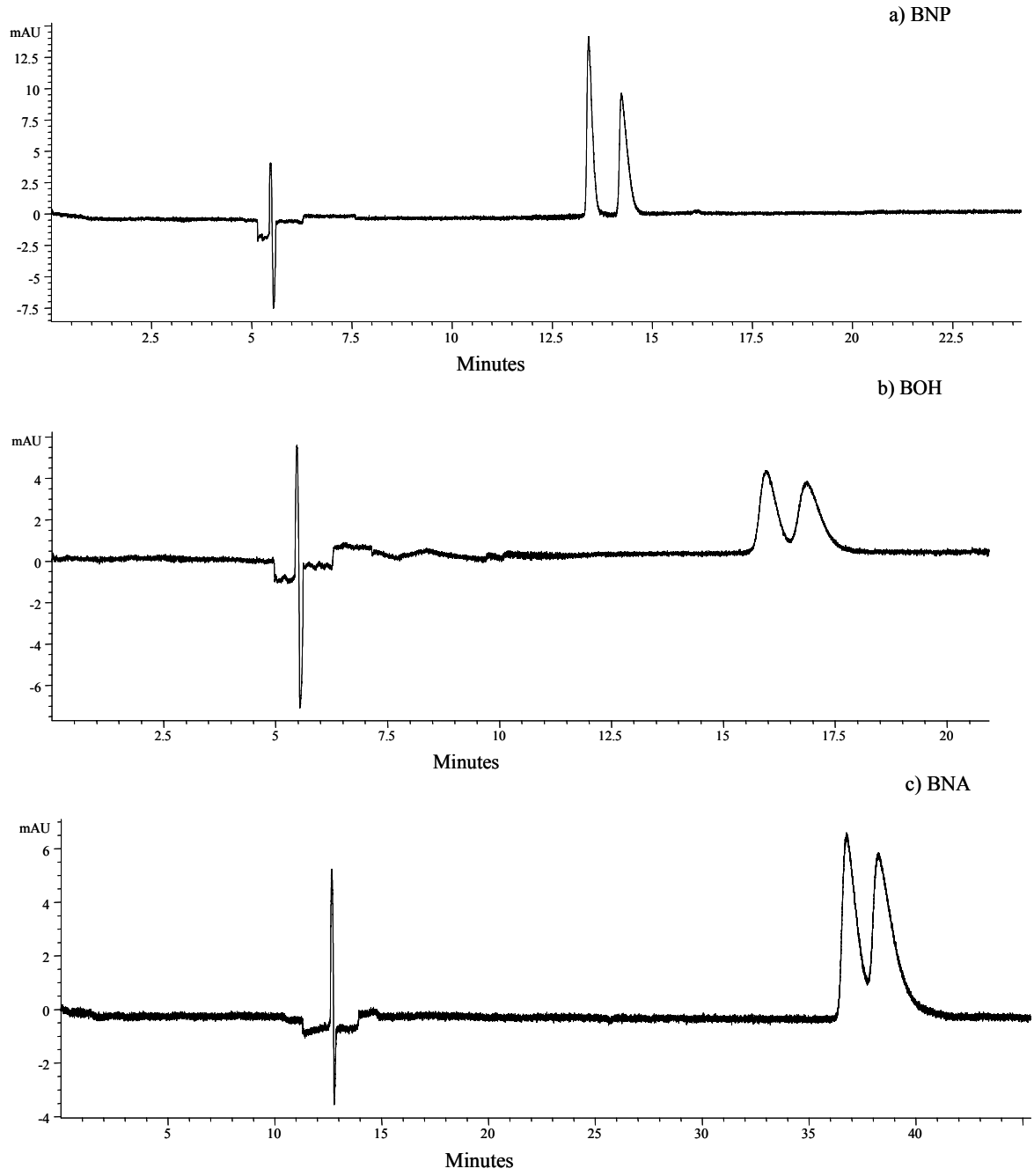


Figure 3.5 Chiral separation of three binaphthyl derivatives. (a) BNP (b) BOH (c) BNA. Conditions: 4 bilayers; 0.02% (w/v) poly-L-lysine with 0.5 M NaCl and 0.25% (w/v) poly-L-SULA; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2), 10% methanol was added to the BGE for the separation of BNA; pressure injection, 30 mbar for 5 s; applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

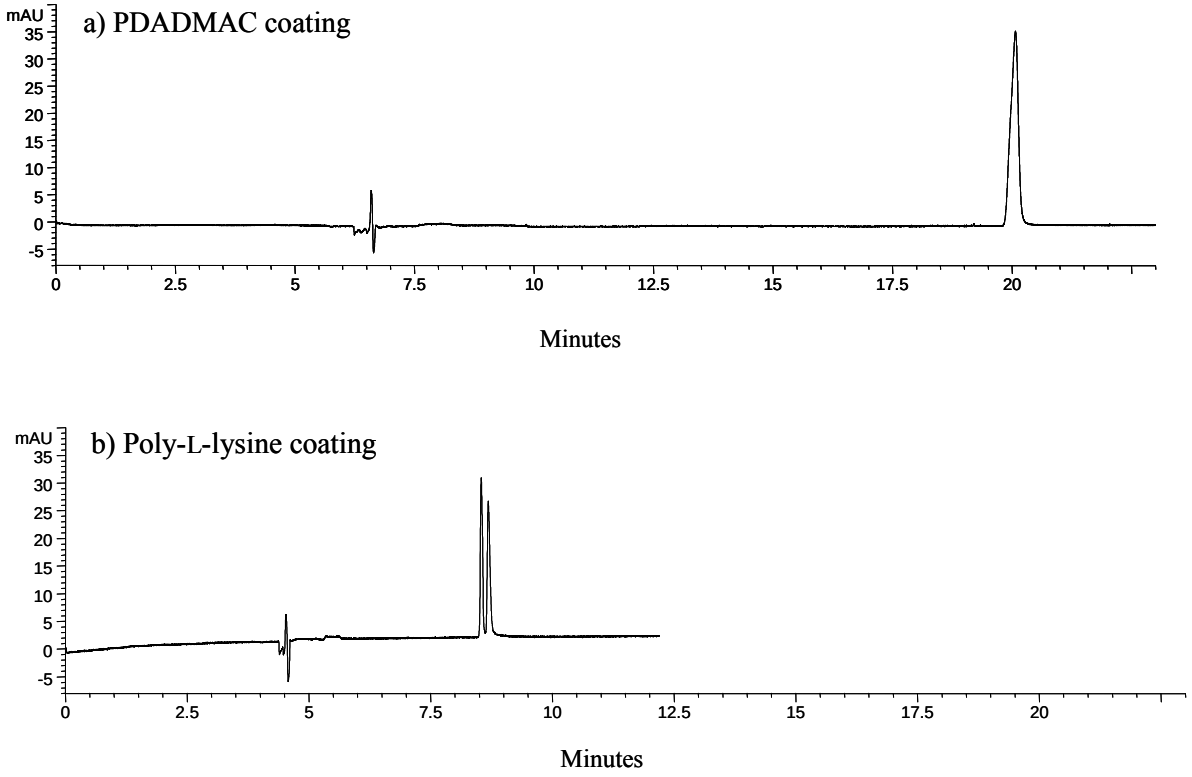


Figure 3.6 Comparison of separation of BNP using (a) 0.02% (w/v) PDADMAC PEM coating (b) 0.02% (w/v) poly-L-lysine Conditions: 1 bilayer; 0.25% (w/v) poly-L-SULA; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); pressure injection, 30 mbar for 5 s; applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

3.5.3 Effect of Voltage

The influence of the applied voltage on resolution, migration time, and efficiency was also investigated for the separation of BOH. Figure 3.8 illustrates the effect of increasing the applied voltage from 15 kV to 30 kV on the separation of BOH. As expected, higher voltages decreased the elution times. At 30 kV the elution time of BOH enantiomers decreased while the peak efficiency increased. On the other hand, when a 15 kV voltage was applied, the resolution increased. It is apparent that at lower voltages the analytes interact more with the PEM coating, and this results in an increase in resolution. Similar observations were made when BNA and labetalol were separated under similar experimental conditions.

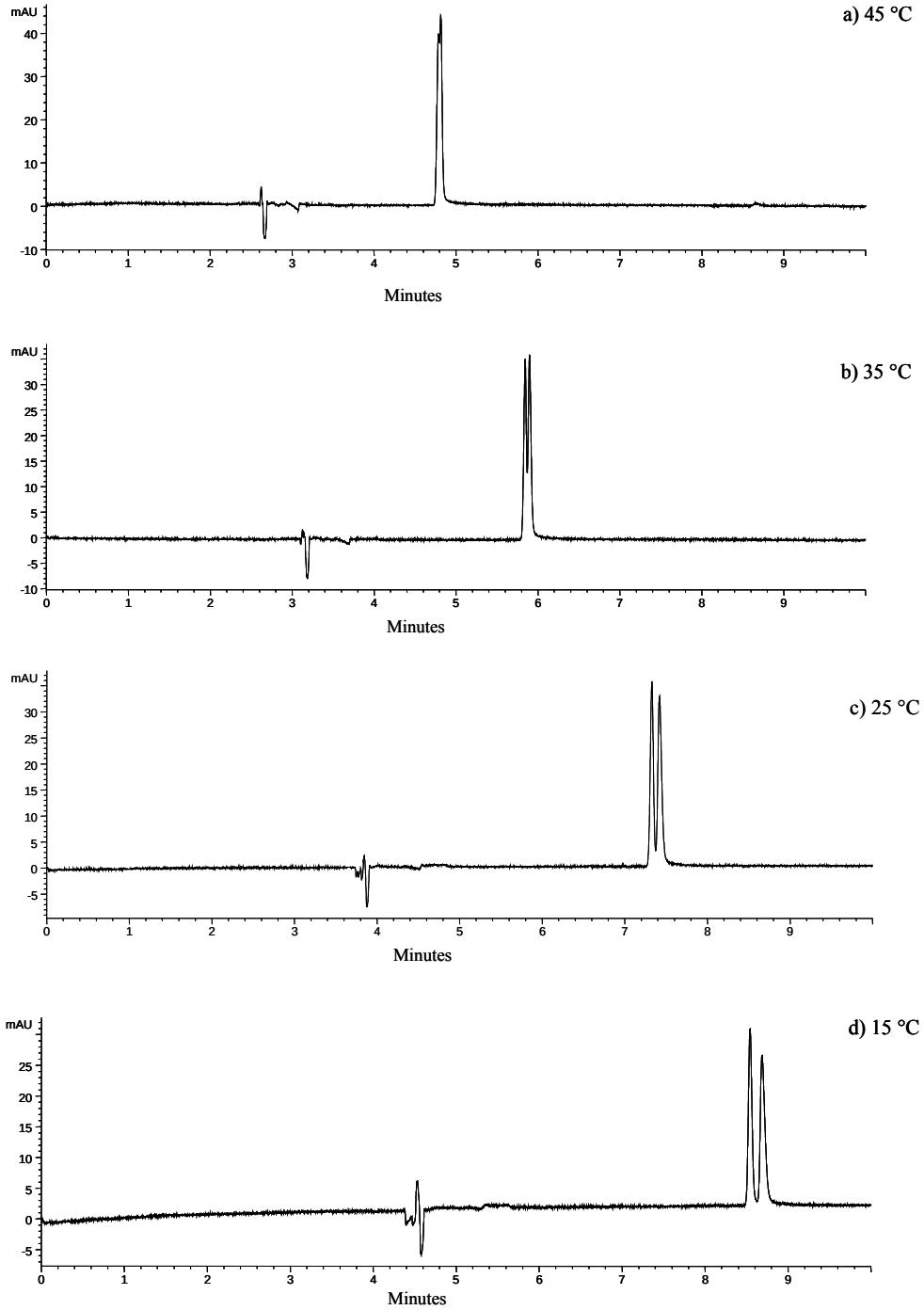


Figure 3.7 Effect of column temperature on the OT-CEC separation of BNP enantiomers. Conditions: 1 bilayer; 0.02% (w/v) poly-L-lysine and 0.25% (w/v) poly-L-SULA; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); pressure injection, 30 mbar for 5 s; applied voltage, 30 kV; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm. (a) temperature 45 °C (b) temperature, 35 °C; (c) temperature, 25 °C (d) temperature, 15 °C.

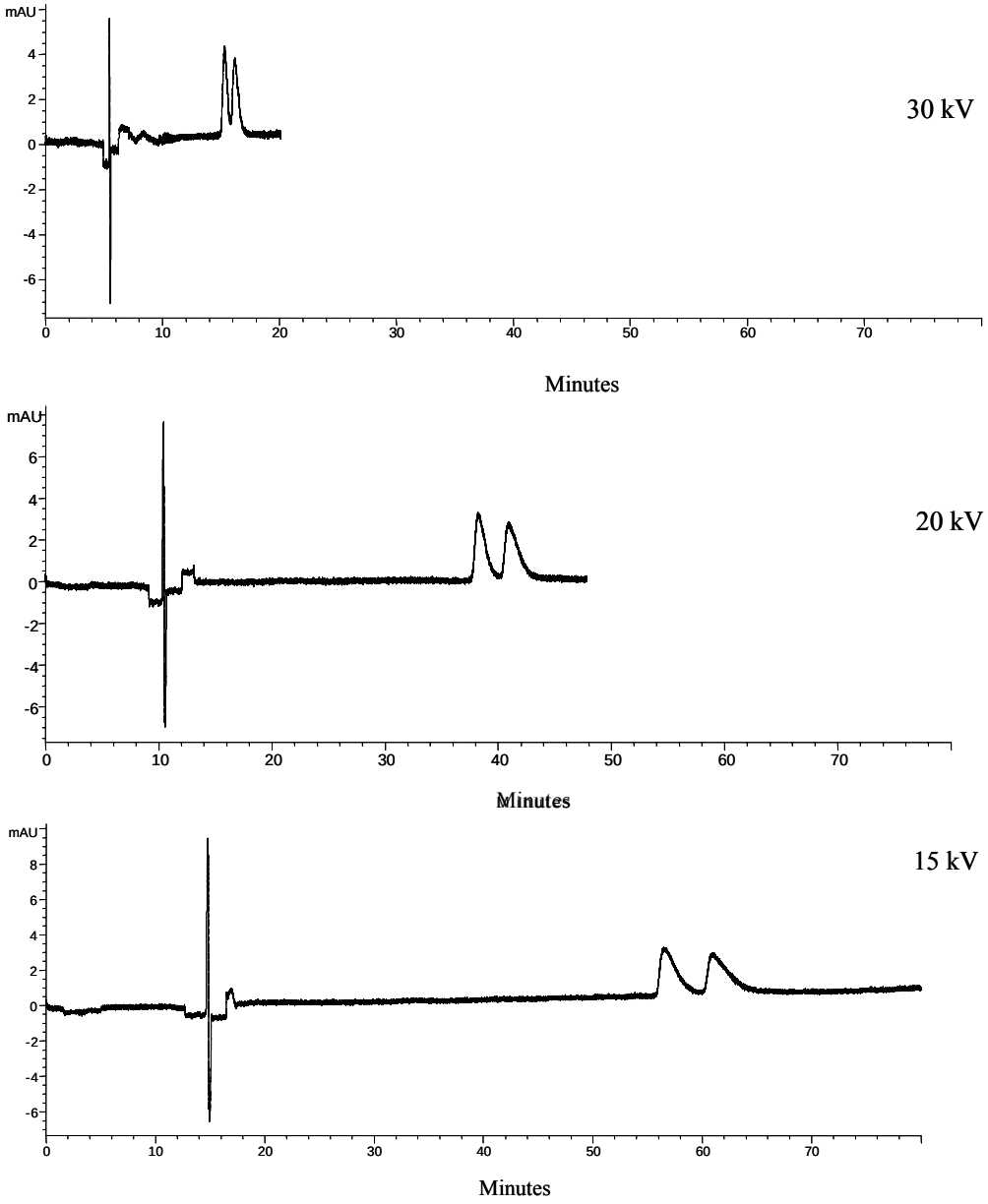


Figure 3.8 Effect of voltage on the migration time and resolution of BOH. Conditions: 4 bilayers; 0.02% (w/v) poly-L-lysine and 0.25% (w/v) poly-L-SULA; 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); pressure injection, 30 mbar for 5 s; BGE, temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm. (a) applied voltage, 30 kV (b) applied voltage, 20 kV (c) applied voltage, 15 kV.

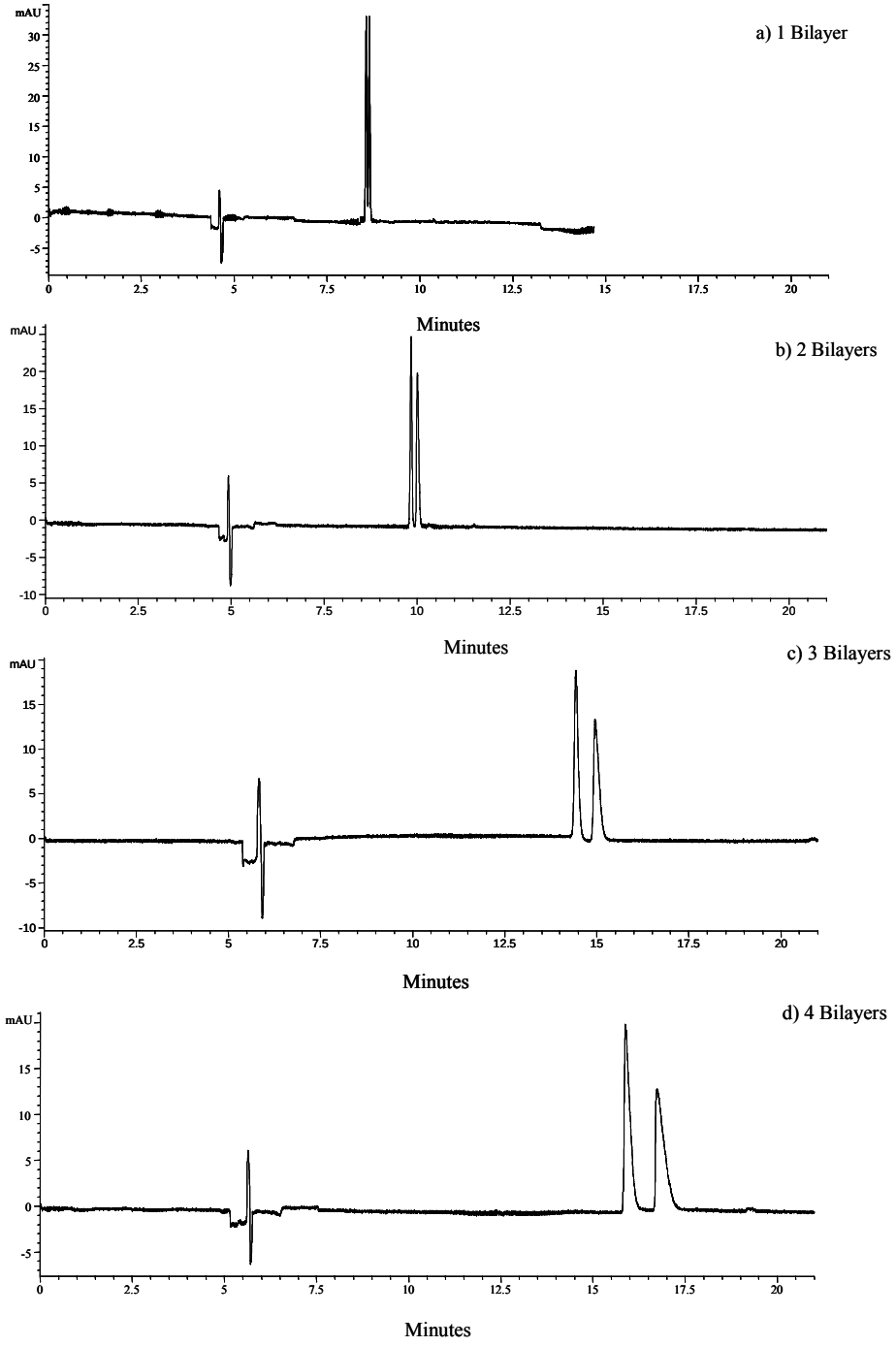


Figure 3.9 Effect of bilayer number on the chiral separation of BNP. Conditions: 4 bilayers; 0.02% (w/v) poly-L-lysine with 0.5 M NaCl and 0.25% (w/v) poly-L-SULA); BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); pressure injection, 30 mbar for 5 s; applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

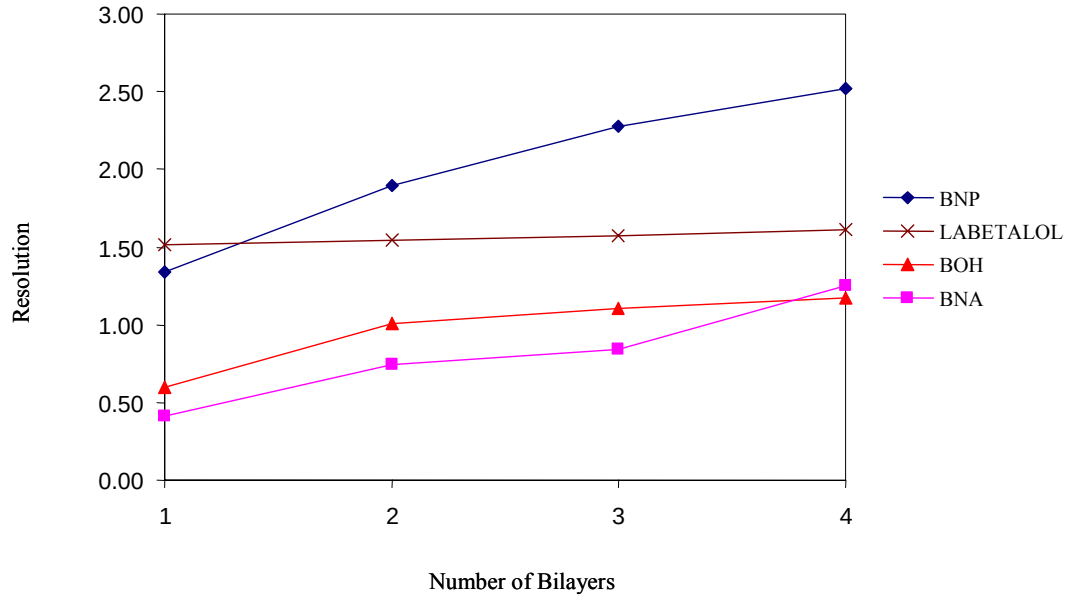


Figure 3.10 Effect of increasing the number of bilayers on the resolution of BNP, labetalol, BOH, and BNA. Conditions same as Figure 3.9

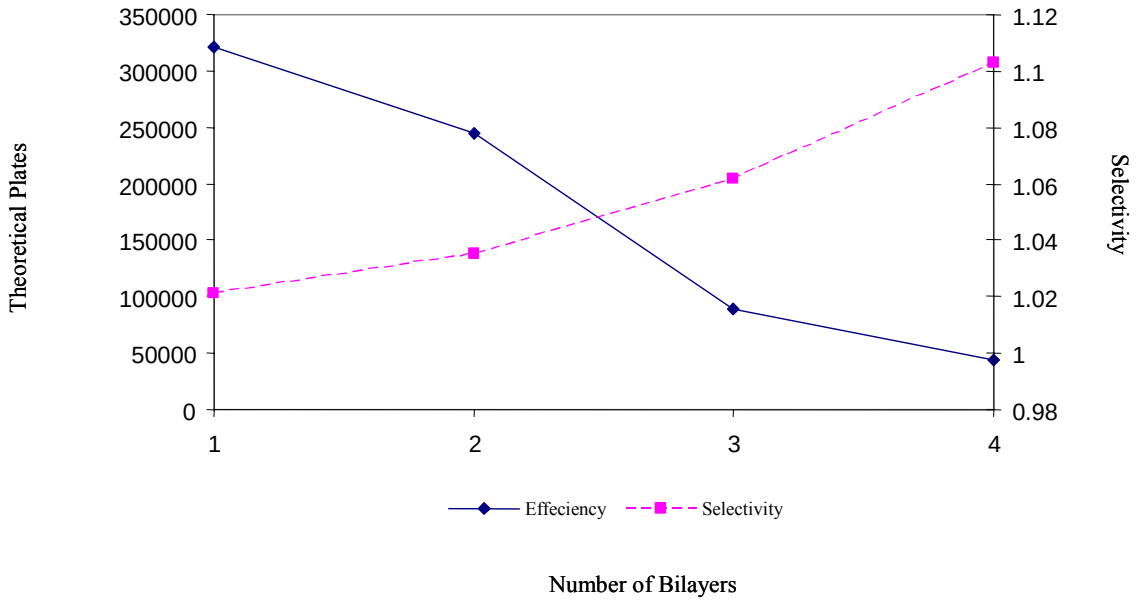


Figure 3.11 Effect of increasing the number of bilayers on efficiency and selectivity of BNP. Conditions same as Figure 3.9

3.5.4 Bilayer Studies

A linear relationship between the thickness of the coating and the number of layers has been illustrated by Dubas *et al*.[37]. In our study, the effect of the number of bilayers on the resolution and selectivity of BNP, BOH, BNA, and labetalol was investigated. The coatings, which were constructed for these experiments, consisted of 1, 2, 3, or 4 bilayers.

As mentioned earlier, a bilayer consists of a layer of a cationic and an anionic polymer. Figure 3.9 illustrates the increase in the enantiomeric resolution of the above analytes as the number of bilayers increases. As the number of bilayers increased, both resolution and selectivity increased, while the peak efficiency decreased. Figure 3.10 illustrates the decrease in the theoretical plates of BNP and the increase in selectivity as the number of bilayers increases. In this study, the 1-bilayer coating gave the highest number of theoretical plates for all four analytes. Thus, these analytes interacted less with the 1-bilayer coating causing less tailing. The peak efficiencies of the BNP enantiomers were 312 679 and 318 706, while those of labetalol diastereoisomers were 256 248 and 262 394 for the 1-bilayer coating.

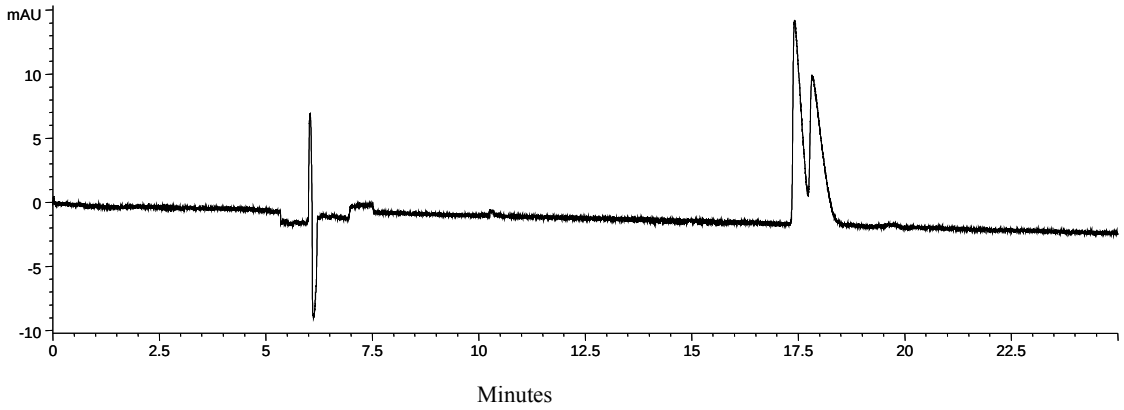


Figure 3.12 Effect of variation of amino acid order on the polymeric dipeptide surfactant on separation of BNP enantiomers. 4 bilayers; 0.02% (w/v) poly-L-lysine with 0.5 M NaCl and 0.25% (w/v) poly-L-SUAL; pressure injection, 30 mbar for 5 s; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

3.5.5 Effect of Variation of Amino Acid Order on Polymeric Dipeptide Surfactant

The influence of the amino acid order in the polymeric surfactant on enantiomeric separations was also investigated. For the purpose of this study, the polymeric surfactants poly-L-SULA and poly-L-SUAL were used as the anionic polyelectrolytes in the construction of the PEM coating. In poly-L-SULA, alanine is the outside (C-terminal) amino acid, while in poly-L-SUAL, it is the inside (N-terminal) amino acid. Figure3.12 illustrates the separation of BNP using poly-L-SUAL. The resolution obtained with poly-L-SUAL was less than 1.5, while the resolution with poly-L-SULA was 2.5 (Figure 3.5a).

A similar observation was made when BOH was separated with poly-L-SUAL. When the chiral selector poly-L-SULA was used, both resolution and selectivity increased. A possible explanation for this observation is that BNP and BOH preferentially interact with the inside (N-terminal) amino acid in each of the polymeric dipeptide surfactants [27]. Thus, the two analytes interact preferentially with the chiral center of leucine in poly-L-SULA and the chiral center of alanine in poly-L-SUAL. Therefore, the decrease in resolution with poly-L-SUAL may be due to the steric hindrance of the butyl group in leucine.

3.5.6 Effect of Sodium Chloride on Selectivity

There are a number of parameters that influence the amount of polymer deposited on a layer during the construction of the PEM coating. These parameters include the molecular weight of the polymer, the deposition time, and the concentration of the salt in the polymer deposition solution. Among these factors, salt has been found to have the greatest influence on the thickness of the coating [37]. For the purpose of this study, the effect of the amount of NaCl in either the cationic or the anionic polyelectrolyte on the separation of different enantiomeric compounds was investigated (Figure 3.13). Figure 3.13a illustrates the chiral

separation of BNP enantiomers obtained when no NaCl was used in the polymer solutions. Baseline resolution at a shorter elution time was obtained. A slight improvement in resolution and an increase in EOF were observed when 0.5 M NaCl was added to the anionic polymer solution (Figure 3.13b). Figure 3.13c was obtained when 0.0 M and 0.5 M NaCl were added to the cationic and anionic polymer solutions, respectively. A significant increase in resolution and a decrease in EOF were observed. Based on the above results, it is possible that the addition of NaCl to the poly-L-lysine solution increases the thickness of the coating more than when NaCl is added to the poly-L-SULA solution. The variation in polymer properties between poly-L-lysine and poly-L-SULA resulted in a variation in the degree of thickness when NaCl is added to each polymer deposition solution. In addition, when NaCl was added to both cationic and anionic polymer solutions, the stability of the current was difficult to maintain. This may have been due to a change in the structure of the PEM coating or an increase in film thickness that can clog the capillary. Based on these observations, higher separation resolution of binaphthyl derivatives can be achieved when NaCl is only added to the cationic polyelectrolyte solution. Studies are ongoing in our laboratory to investigate the thickness and the structure of the PEM coating when NaCl concentration in the PEM coating is varied.

3.5.7 Column Reproducibility and Stability Studies

Both reproducibility and stability are very important factors for evaluating the performance of the coating. Figure 3.14 is an illustration of the remarkable run-to-run reproducibility of the 5^{th}, 10^{th} and 15^{th} runs for the separation of labetalol. In this study, a BGE of 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2) was used, and no NaCl was added to the polymer deposition solutions. These results were reproducible in five different capillaries with run-to-run RSD

values (n = 5) of 0.92%, 0.43%, 0.93%, 0.70% and 0.85%. In order to assess the capillary-to-capillary reproducibility, five capillaries were prepared under similar conditions. The RSD values of the EOF were computed from five consecutive runs for each coated capillary. All values were found to be less than 1%.

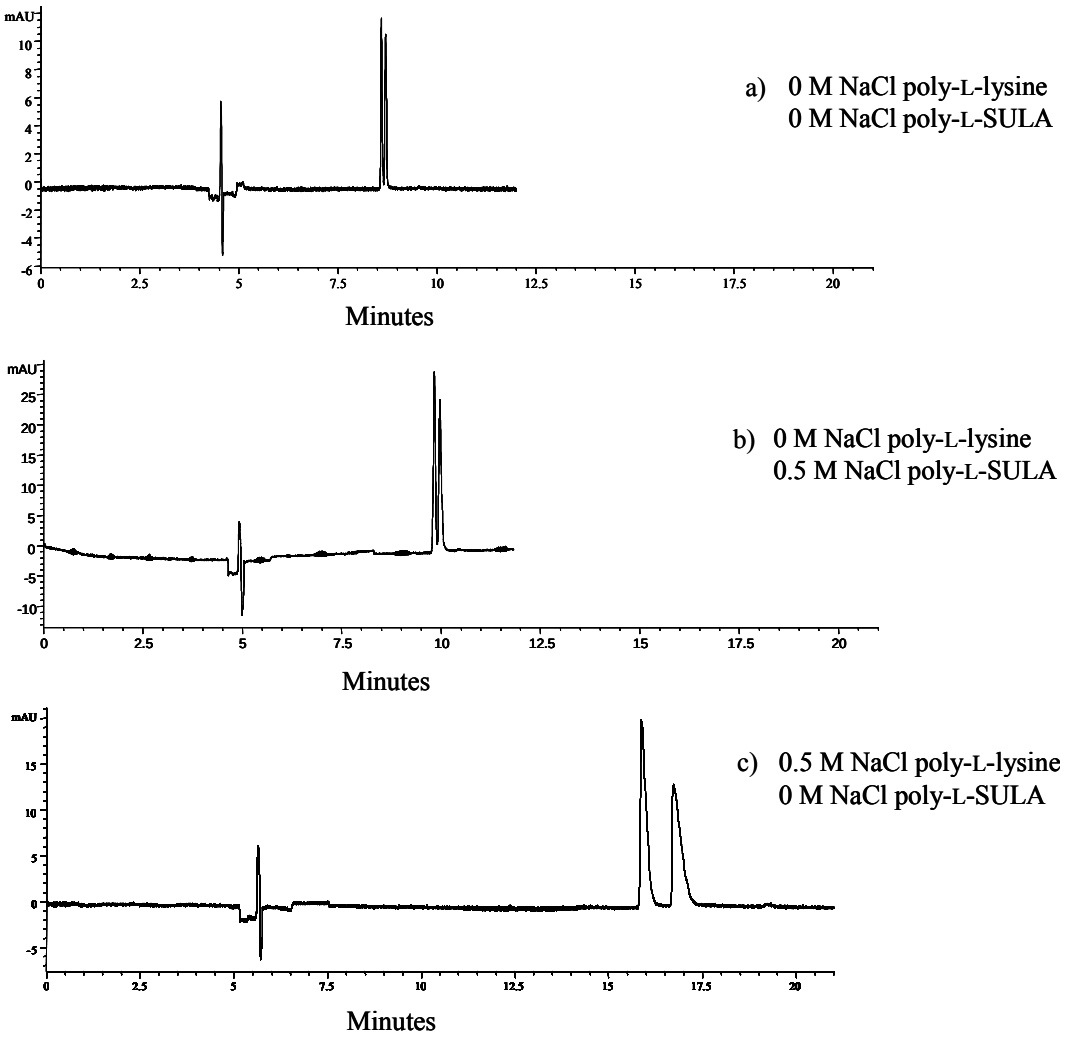


Figure 3.13 Effect of NaCl concentration on the chiral separation of BNP. Conditions: 4 bilayers; pressure injection, 30 mbar for 5 s; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm. a) 0.02% (w/v) poly-L-lysine with 0 M NaCl and 0.25% (w/v) poly-L-SULA with 0 M NaCl. (b) 0.02% (w/v) poly-L-lysine with 0.0 M NaCl and 0.25% (w/v) poly-L-SULA with 0.5 M NaCL. (c) 0.02% (w/v) poly-L-lysine with 0.5 M NaCl and 0.25% (w/v) poly-L-SULA with 0.0 M NaCl.

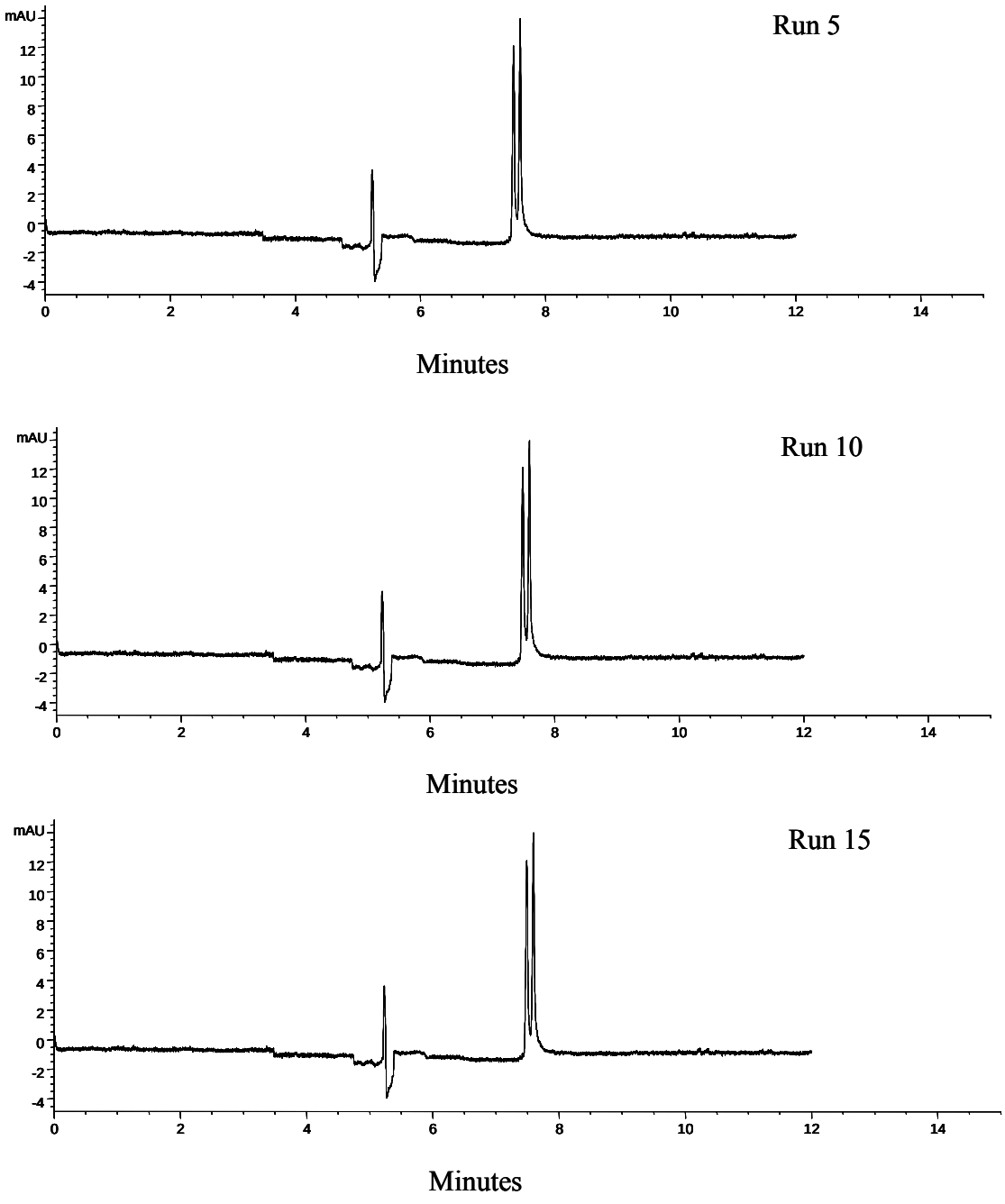


Figure 3.14 Illustration of the run-to-run reproducibility for the chiral separation of labetalol. Conditions: 4 bilayers; 0.02% (w/v) poly-L-lysine and 0.25% (w/v) poly-L-SULA; BGE, 100 mM Tris and 10 mM $Na_2B_4O_7$ (pH 10.2); pressure injection, 30 mbar for 5 s; applied voltage, 30 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

The endurance of the coating was evaluated by performing a consecutive number of runs in the same capillary over a period of five days. The capillary was conditioned with a fresh BGE after every 15 runs. The PEM-coated capillary endured over 290 runs. It was also observed that the capillaries coated with no NaCl added to the polymer solutions gave the best reproducibility of both EOF and analyte migration times.

3.5.8 Separation of β-blockers

The drug labetalol is a β-blocker that is used for the treatment of hypertension [46]. This compound has two chiral centers and therefore four diastereoisomers (four peaks). However, only two diastereoisomers were resolved in this work (Figure 3.14). The separation of sotalol is illustrated in Figure 3.15 and it elutes before the EOF since it is cationic under the experimental conditions used in this study. The second peak, which is of low efficiency, is typical for the chiral separation of β-blockers. This may be due to the higher affinity of one of the enantiomers to the chiral PEM coating.

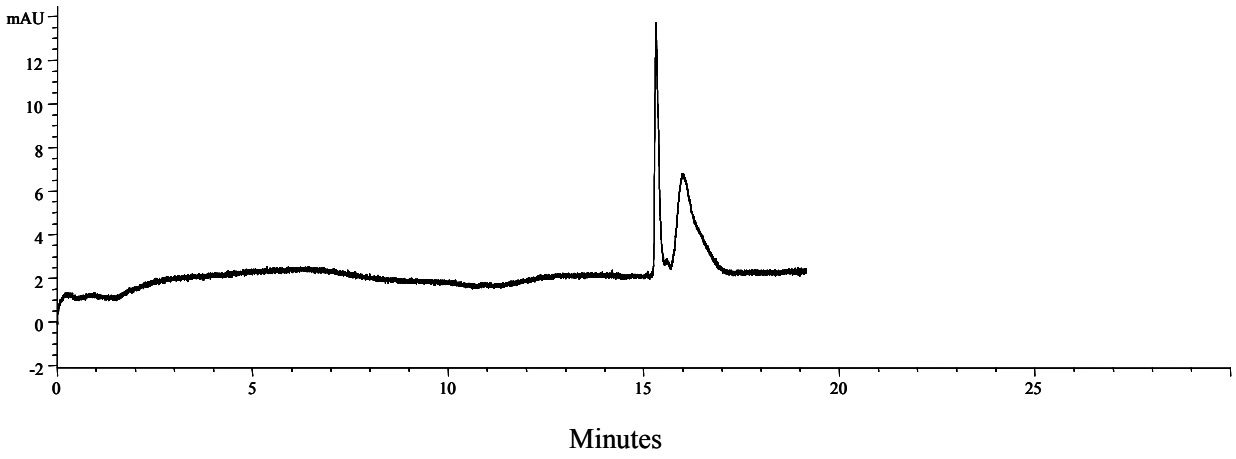


Figure 3.15 Chiral separation of sotalol. Conditions: 4 bilayers; 0.5% (w/v) poly-L-lysine) and 0.5% (w/v) poly-L-SULA with 0.1 M NaCl; pressure injection, 30 mbar for 3 s; BGE, 300 mM CAPs and 50 mM $Na_2B_4O_7$ (pH 8.5), 0.15% hexylamine; applied voltage, 20 kV; temperature, 15 °C; capillary, 57 cm (50 cm effective length) × 50 μm i.d.; detection, 220 nm.

The use of hexylamine in the mobile phase has been shown to improve the chiral selectivity of β-blockers [47]. Therefore in this study, 0.15% (v/v) hexylamine was added to the BGE in order to improve the resolution between the enantiomers.

3.5.9 Coupling of OT-CEC to MS

The use of the PEM coating with MS is beneficial for the coupling of MEKC to MS. In this study, we investigated the use of the PEM coated capillary for the chiral separation of labetalol. A number of parameters were varied in order to make the PEM coated capillary amenable to coupling with MS. The separations performed for this study required the use of a purely aqueous electrophoresis medium. Thus, ammonium acetate was used as the BGE. In addition, the total capillary length was increased to 61 cm in order to facilitate the coupling of the Hewlett-Packard ^{3D}CE instrument to the MS. The total ion chromatogram (TIC) and mass spectra for the separation of labetalol diastereoisomers is illustrated in Figure 3.16. Although a baseline separation was not obtained, it was possible to identify the parent ions of the diastereoisomers in the mass spectrum.

3.6 Conclusions

A novel PEM coating has been applied for chiral separations in OT-CEC. Several factors have been shown to affect the chiral selectivity. The presence or absence of NaCl in each of the polyelectrolytes has been shown to play a significant role in the selectivity of the chiral PEM coating. A 4-bilayer coating was found to be optimal for the separation of the 3 binaphthyl derivatives BNP, BOH, and BNA. The separation of BNP enantiomers and labetalol diastereoisomers resulted in high peak efficiencies with theoretical plates of over than 250 000. Run-to-run and capillary-to-capillary reproducibility were very good, and the

RSD values of the EOF were less than 1%. The coating endured over 290 runs. In addition, the mass spectrometric detection of labetalol is accomplished by coupling OT-CEC to MS.

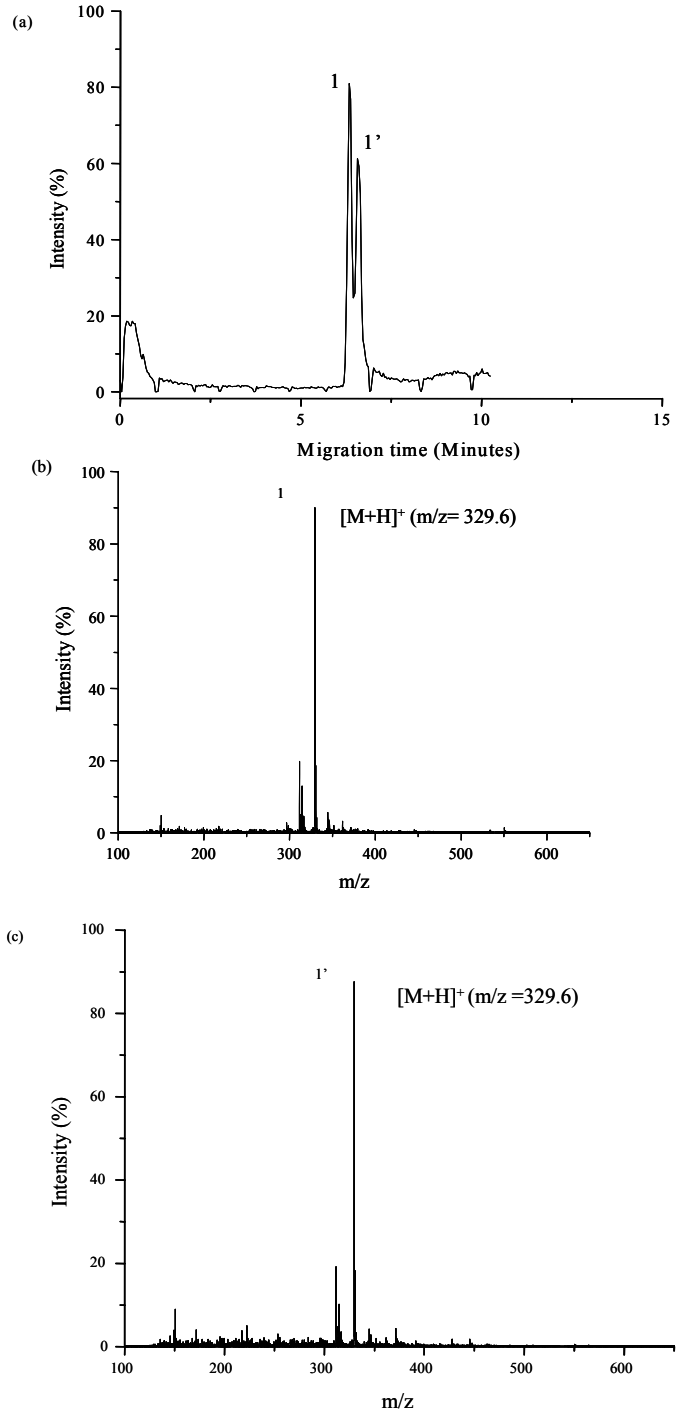


Figure 3.16 OT-CEC/MS of labetalol diastereoisomers. (a) Total ion chromatogram (b) Mass spectra. Conditions: 4 bilayers; 0.02% (w/v) poly-L-lysine and 0.25 % (w/v) poly-L-SULA; BGE, 10 mM NH_4OH (pH 9.0); pressure injection, 50 mbar for 5 s applied voltage, 20 kV; capillary, 61 cm × 50 μm i.d.

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CHAPTER 4

OPEN-TUBULAR CAPILLARY ELECTROCHROMATOGRAPHY/ELECTROSPRAY IONIZATION MASS SPECTROMETRY USING A POLYELECTROLYTE MULTILAYER COATING

4.1 Introduction

Interfacing capillary electrophoresis (CE) with mass spectrometry (MS) is of growing interest in separation science because CE offers rapid separation, high separation efficiency and small sample consumption, while MS is capable of providing mass and structural information for analytes of interest. Among the CE separation modes, capillary zone electrophoresis (CZE), where separation ability is based on the analyte's mass to charge ratio is the most frequently used due to its simplicity. Unlike CZE, both micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) have a wide application range because of their ability to separate both neutral and charged analytes. In both MEKC and CEC, the separation is achieved by a combination of the electrophoretic mobility difference of analytes and the partitioning interaction between the analytes and the pseudostationary or stationary phase. However, coupling MEKC with MS can be challenging due to the negative effect of the background surfactants on the mass signal. In electrospray ionization mass spectrometry (ESI MS), nonvolatile surfactants such as sodium dodecyl sulfate (SDS), which are present in the running buffer at relatively high concentrations, produce low ionization efficiency. This causes suppression of the analytes' mass signal and mass detector contamination [1-3].

In order to overcome the deliterious effects of surfactants when CE is couple to in ESI MS, Varghese and Cole used a cationic surfactant cetyltrimethylammonium chloride at low concentration for the analysis of cationic tripeptides and other amine-containing compounds

[4] . However, in this case the separation mode employed was not MEKC because the concentration of added surfactant was below its critical micellar concentration (CMC). In recent years, the use of polymeric surfactants in MEKC has received much attention [5-10]. As compared to monomeric surfactants, polymeric surfactants have the advantages of a zero CMC and negligible surface activities. The first attempt of direct coupling of MEKC with ESI MS was reported by Ozaki et al. [11]. In this study, the use of a high molecular weight polymeric surfactant with zero CMC made it possible to form a micelle at a very low surfactant concentration, thus reducing the interference of surfactants in ESI MS. Lu and co-workers also developed a polymeric surfactant, poly (sodium undecenyl sulfate) (poly-SUS), for the resolution enhancement of the analytes in MEKC/ESI MS [12].

Although the use of polymeric surfactants can decrease the interference of the surfactant on mass signal, problems still remain. During the ionization process, the polymeric surfactant can still be introduced into the MS and the analyte-surfactant adduct mass signal may be observed [13]. In addition, the analyte and analyte-surfactant complex may have different ionization efficiencies due to the significant difference in molecular size [14]. As a result, formation of the analyte-surfactant complex may lower the ionization efficiency of the analyte, and reduce the sensitivity of MS detection by use of MEKC/ESI MS.

Alternative methods have been developed to eliminate the introduction of pseudostationary phase into the MS. Foley and Masucci [15] used a semipermeable membrane that only allowed small molecules to permeate the membrane while retaining large molecules. Nelson et al. [16] interfaced partial-filling MEKC with ESI MS where the capillary was filled with a small plug of running buffer that containing a micellar solution. The analytes first migrated into the micellar plug where they were separated, and then into

the running buffer before entering the mass spectrometer. In another report, Yang et al. reversed the migration direction of the micelle such that it was opposite to the direction of the analytes. This was achieved by adjusting the pH value of the MEKC buffer [17]. It is apparent that either partial-filling or opposite migration method is only applicable to select analytes in MEKC/ESI MS, and the stability of those methods still remains questionable [18].

The stationary phase for open-tubular CEC (OT-CEC) is immobilized on the inner surface of the capillary unlike MEKC where the pseudostationary phase is directed added into the running buffer. In comparison with packed CEC, where the stationary phase is packed in the column, OT-CEC does not suffer from the air bubble formation problem which exists around the frits and packing materials in packed columns. Moreover, the running buffer in OT-CEC can be replenished between each run by simply flushing the capillary with fresh solution. Hence, it is an almost ideal separation technique that can be used for coupling with ESI MS. Wu et al. prepared a reversed-phase OT-CEC column coupled with MS for ultrafast analysis of a peptide mixture [19]. After covalently binding the reversed-phase C-8 on the capillary wall, an amine group was coated onto the C-8 surface to increase the electroosmotic flow (EOF). However, the column preparation was tedious and time-consuming. Different kinds of coatings, such as polyelectrolytes and ionic liquids have also been used for capillary wall modification [20-23]. However, unlike the OT-CEC coatings, such coatings on the capillary inner wall are primarily used for the purpose of reducing capillary wall interactions with the analytes.

Previous studies in our laboratory have investigated the use of the polymeric surfactant, poly (sodium N-undecenyl-L-glycinate) as a stationary phase coating in OT-CEC for the

separation of benzodiazepines [24] . The polymeric surfactant was successfully immobilized on the capillary wall using a polyelectrolyte multilayer (PEM) coating. The column was alternatively flushed with the cationic polymer poly (diallyldimethylammonium chloride), PDADMAC, and the anionic polymeric surfactant solution, each for ten times. The ten-bilayer coating resulted in the formation of a stable coating due to the strong electrostatic attraction between layers; however, a drawback to this approach is that the multiple coating steps may be time-consuming. In a subsequent study, the anionic polymeric surfactant, poly (sodium undecenyl sulfate), poly-SUS, and cationic PDADMAC were investigated for the formation of a single bilayer coating on the capillary inner wall [25]. The OT-CEC column demonstrated good stability and great separation performance. In addition, when comparing the previously used polymeric surfactant, poly-SUS is more suitable as it can be used over a wider pH range.

In this chapter, the use of poly-SUS as a stationary phase coating in OT-CEC/ESI MS is investigated for the first time. The separation of β-blocker and benzodiazepine analytes is studied. This technique is combines the favorable aspects of MEKC and OT-CEC to enable MS detection.

4.2 Experimental

4.2.1 Reagents and Chemicals

Glacial acetic acid, methanol, and acetone of HPLC grade were purchased from Fisher Scientific (Fair Lawn, NJ). Ammonium hydroxide was purchased from Mallinckrodt Baker (Paris, KY). Ultra pure grade ammonium acetate was purchased from Amresco Inc. (Solon, OH). Cationic polymer PDADMAC, with a molecular weight range of 200,000 to 350,000,

was obtained from Aldrich Chemical (Milwaukee, WI). The anionic poly-SUS was synthesized according to our previously reported procedure in Chapter 2, Section 2.2.2.

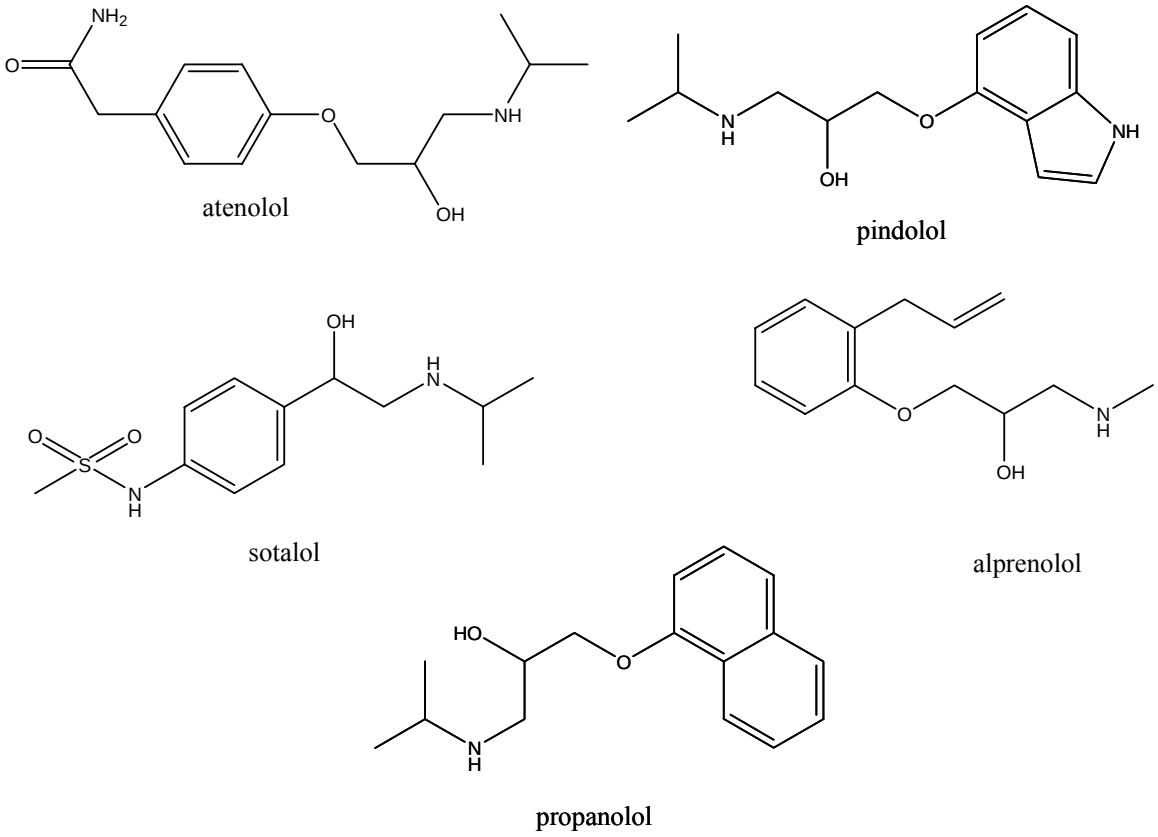

Figure 4.1 Chemical structures of β-blockers investigated.

Chlorosulfonic acid, 10-undecenyl alcohol, and the analytes; alprenolol, atenolol, pindolol, propranolol, sotalol, clonazepam, flunitrazepam, lorazepam, nitrazepam were purchased from Sigma (St. Louis, MO). Chemical structures of β-blocker analytes are shown in Figure 4.1, while those of the benzodiazepines are shown in Figure 2.4 (Chapter 2). Deionized water used in the preparation of all solutions was obtained from an USFilter system (Lowell, MA). All other chemicals were analytical grade. The bare fused-silica capillary 50 μm i.d., 360 μm o.d. was purchased from Polymicro Technologies (Phoenix, AZ).

4.2.2 Buffer and Sample Preparation

The running buffer solution for OT-CEC/ESI MS containing 10 mM ammonium acetate was adjusted to pH 4.0 and 6.0 with acetic acid, and to pH 9.0 with ammonium hydroxide. The solution of the sheath liquid consisted of 0.5% acetic acid in 50:50 methanol/water (v/v). Before use, each solution was sonicated for 10 min, and then filtered with 0.45 μm syringe filter (Whatman, Clifton, NJ). Stock standard solutions of analytes were prepared by dissolving each compound in methanol to obtain a concentration of 1 mg/mL. All solutions were stored at 4 °C before use. For OT-CEC/ESI MS experiments, aliquots of analyte stock solution were diluted in methanol to 0.1 mg/mL, and samples for injection were prepared by further dilution with running buffer.

4.2.3 Procedure for Polyelectrolyte Multilayer Coating

The OT-CEC column was prepared by using the PEM coating procedure described in Section 2.5 (Chapter 2). Briefly, the capillary was preconditioned by rinsing with 1 M NaOH for 45 min in order to enhance the deprotonation of the silanol groups, followed by a 15-min rinse with deionized water. After preconditioning, the capillary was rinsed with the solution containing 0.5% w/v PDADMAC in 0.2 M NaCl for 20 min to deposit a cationic polymer layer. Then, the capillary was rinsed with deionized water for 5 min. Finally, 1% w/v poly-SUS solution was rinsed over the cationic layer for 20 min to immobilize the polymeric surfactant coating on the internal surface of the capillary. Any residual poly-SUS was removed by rinsing the capillary with deionized water for 5 min. The length of the poly-SUS coated capillary was 61.5 cm for this experiment.

4.2.4 Instrumentation

An Hewlett-Packard ^{3D}CE instrument (Palo Alto, CA) coupled to time of flight mass spectrometer (TOF MS) Mariner Biospectrometry Workstation from Applied Biosystem (Framingham, MA) was employed for OT-CEC/ESI MS experiments. All experiments were performed at room temperature. A CE ESI sprayer from Agilent Technologies (Palo Alto, CA) was used to interface the CE with the MS and was capable of providing both a coaxial sheath liquid and a nebulization gas to assist the electrospray as shown in Figure 4.2. The capillary tip was set at an angle of 45 degrees relative to the direction of the ESI TOF MS nozzle in order to obtain optimum signal.

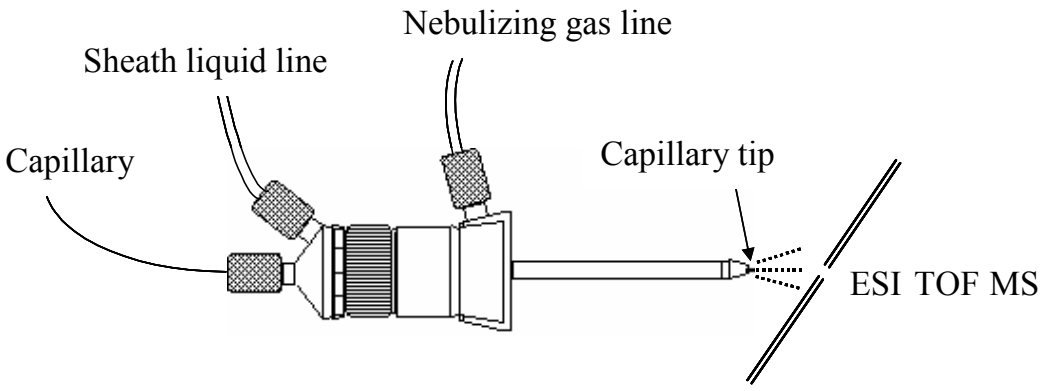

Figure 4.2 Schematic representation of the sheath flow interface used illustrating the sheath liquid and nebulizing gas for electrospray formation.

The sheath liquid was delivered at a flow rate of 4 μL/min by use of a Harvard Apparatus syringe pump (Holliston, MA) while the ESI voltage was set at 3.5 kV in the positive mode. Data acquisition was performed in the range of m/z 50-1000 at a scan rate of 3 s per spectrum. The nebulizer and curtain gases were both nitrogen, and the flow rates were optimized at 0.6 L/min and 0.7 L/min, respectively. Between runs, the capillary was rinsed

with the running buffer for 2 min. A pressure 50 mbar for 5 s was applied for sample injection.

4.3 Results and Discussion

In OT-CEC, the stationary phase is immobilized on to the capillary wall and it enhances the separation of analytes by a partitioning interaction. In this study, a PEM consisting of the cationic PDADMAC and the anionic poly-SUS, was physically adsorbed on to the capillary wall and used as a stationary phase for OT-CEC separations. For a bare fused-silica capillary, the EOF was generated as a result of the deprotonated silanol groups on the inner surface, as summarized in Table 4.1. For CZE separations, EOF direction and magnitude were related to the type of surface charge and the surface charge density, respectively. After flushing with the cationic PDADMAC, the EOF direction was reversed resulting in a different magnitude as compared to that of a bare fused-silica capillary. The results in Table 4.1 indicated that the cationic polymer was adsorbed on the inner surface and the overall surface charge was positive. After a continuous rinse with the anionic polymeric surfactant, poly-SUS, the EOF change in terms of both direction and magnitude confirmed that the anionic poly-SUS successfully immobilized on the PDADMAC layer, as expected in the formation of a PEM coating.

Table 4.1 EOF magnitude measured after each polymer deposition on fused silica capillary

Capillary	EOF (m^2/s.v) × 10^{-8})
Bare fused silica	3.86
Coated with PDADMAC	-2.28
Coated with PDADMAC/Poly-SUS	2.88

The pH of the OT-CEC running buffer plays a crucial role in the separation of analytes because it affects not only the charge of the analyte, but the charge of the stationary phase. The influence of pH on the separation of five β-blocker analytes was investigated as shown in Figure 4.3. At pH 4.0, the basic analytes carry positive charges. In addition to a partitioning interaction between the analyte and the hydrophobic core of poly-SUS immobilized on the capillary wall, the positively charged analytes have a strong electrostatic interaction with the sulfonate groups on the polymeric surfactants, thus leading to long migration times of the analytes and to broad peaks. When the pH of the buffer is increased, the positive charges of the analytes decrease, and therefore, the electrostatic interaction between the analytes and the polymeric surfactant is weakened. In addition, the EOF is increased with increasing buffer pH. In this case, both the molecular interaction and the increased EOF resulted in enhanced separation at pH 9.0. At this pH, the separation of the five analytes using the polymeric surfactant-coated capillary was improved as compared to the separation using a bare fused-silica capillary.

The influence of applied voltage, ranging from 20 to 30 kV, on the separation was also investigated. As indicated in Figure 4.4, the peak efficiency improved upon increasing the voltage from 20 to 30 kV, and the migration time is decreased accordingly. Better peak resolution was achieved at a higher applied voltage; however, the effect of applied voltage on analyte separation did not have as significant an effect as varying the buffer pH.

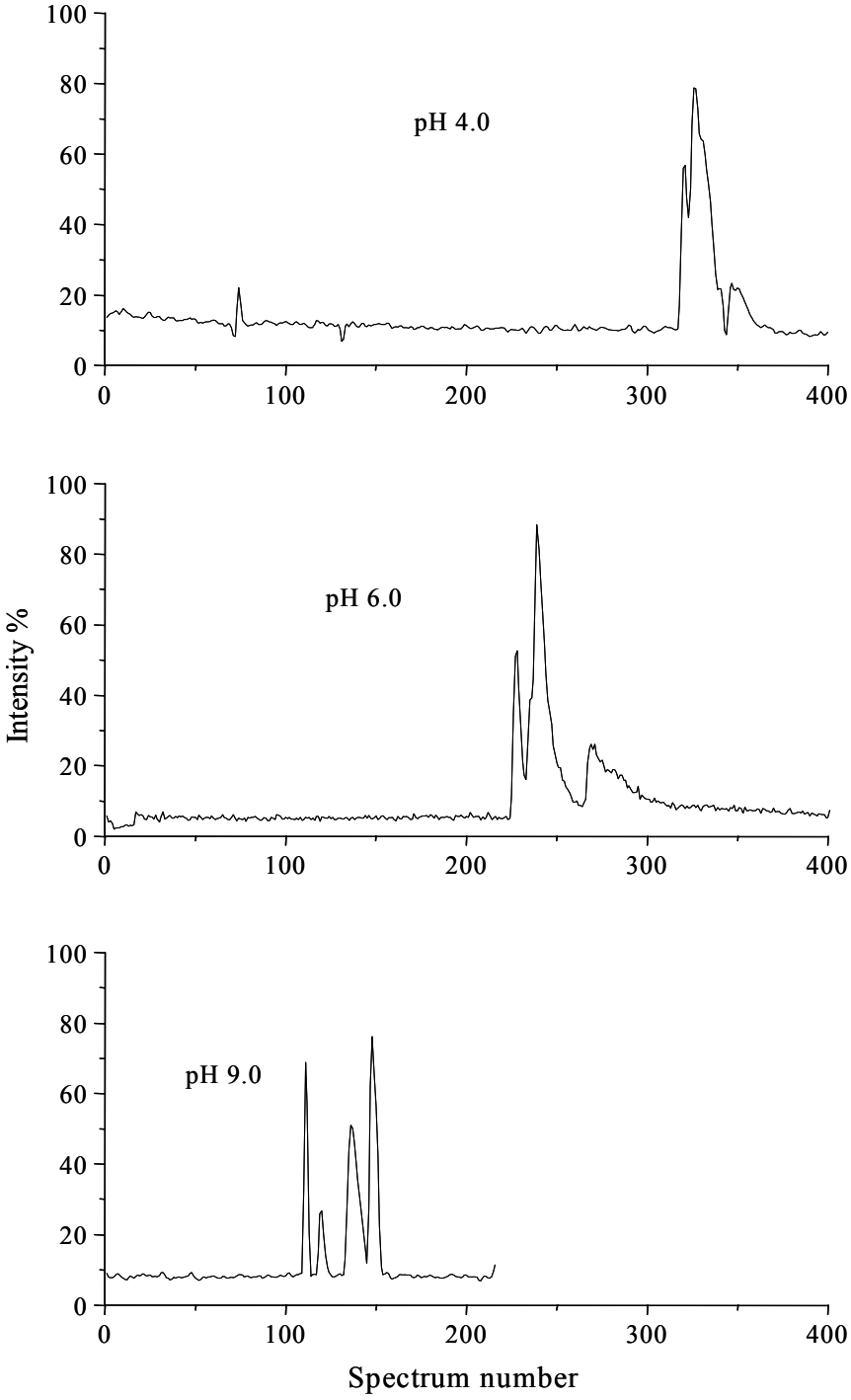

Figure 4.3 Effect of buffer pH on the separation of β-blockers in OT-CEC/ESI MS. Conditions: PEM coating; 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; Buffer: 10 mM NH_4Ac; CE separation voltage: 25 kV; capillary: 61.5 cm total length, 50 μm i.d.

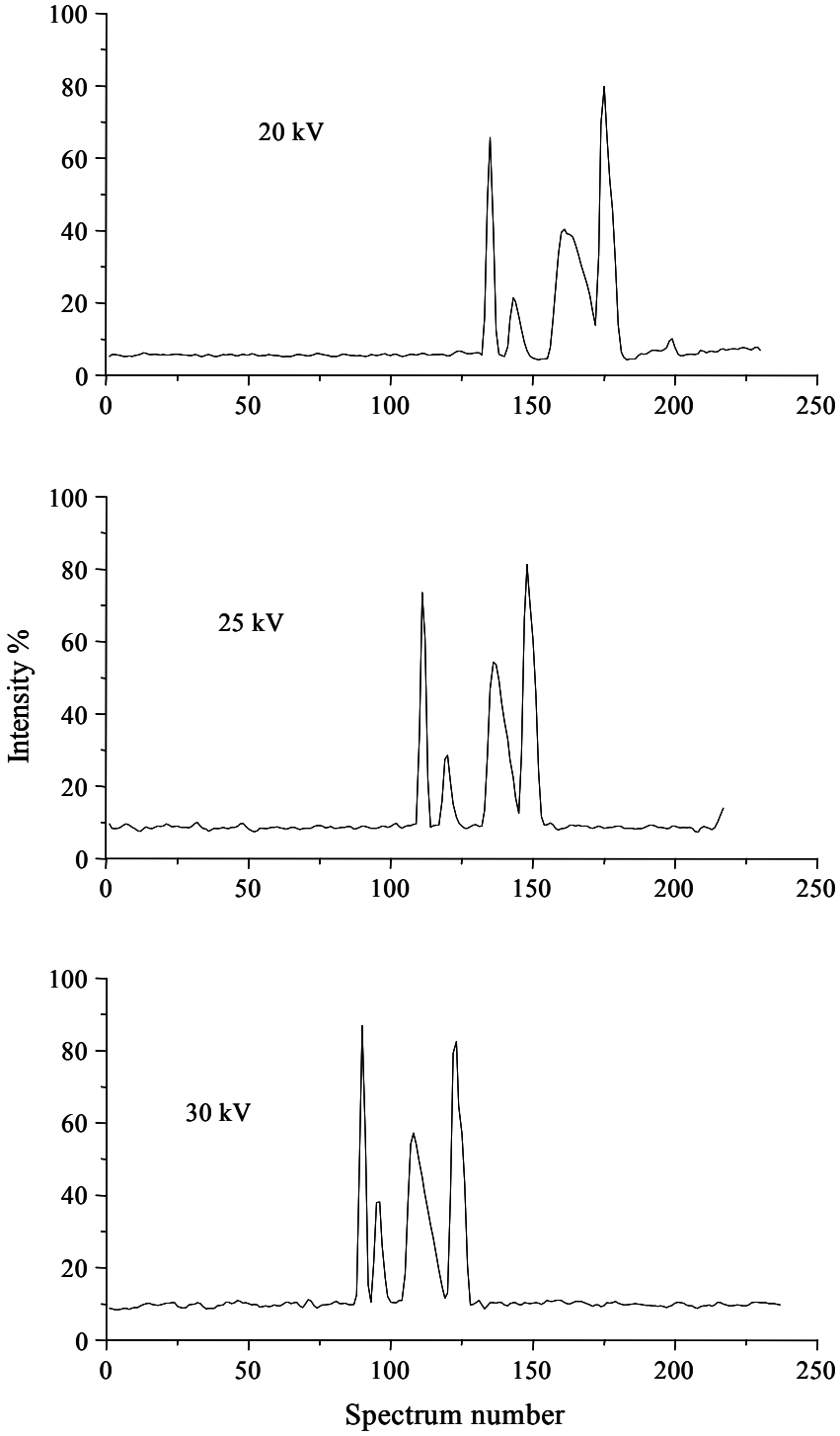

Figure 4.4 Effect of CE separation voltage on the separation of β-blockers at pH 9.0 in OT-CEC/ESI MS. For other conditions see Section 4.2.4. Conditions: PEM coating; 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; Buffer: 10 mM NH_4Ac; CE separation voltage: 25 kV; capillary: 61.5 cm total length, 50 μm i.d.

The selected ion chromatograms (SIC) and mass spectra obtained from OT-CEC/ESI MS are shown in Figure 4.5 and Figure 4.6 respectively. Under the optimum conditions, four of the five β-blockers were separated. The migration order of the analytes can be determined according to their m/z signals without the use of standards that are necessary for peak identification when only a UV-vis detector is employed. Under the selected separation conditions, sotalol co-eluted with alprenolol while the latter analyte had a broad peak with lower peak efficiency. Among the five analytes, the propranolol structure has a biphenyl group and thus, a higher hydrophobicity. Furthermore, the analytes have similar pK_a values. Therefore, the long migration time of propranolol may result from stronger hydrophobic interactions with the core of poly-SUS. In the mass spectra of the analytes, analyte-potassium adducts were also found. However, the intensity of adducts differed between analytes.

Another successful application of OT-CEC/ESI MS was the separation and identification of four benzodiazepines. The SIC and mass spectra are shown in Figures 4.6a and 4.6b, respectively. Baseline separation is achieved for the four analytes; however, clonazepam and lorazepam had low ionization efficiency, as shown in Figure 4.6a. In addition to the analyte-potassium adduct signal, the chlorine isotope mass signals, which have 2 m/z unit differences, were also observed clonazepam and lorazepam (Figure 4.7b). The four benzodiazepine analytes failed to be separated from each other and eluted as a single peak when a bared fused-silica capillary was used (Figure 2.10a, Chapter 2). These results indicate that the poly-SUS based PEM coating in OT-CEC is very important for baseline separation of the four benzodiazepine analytes.

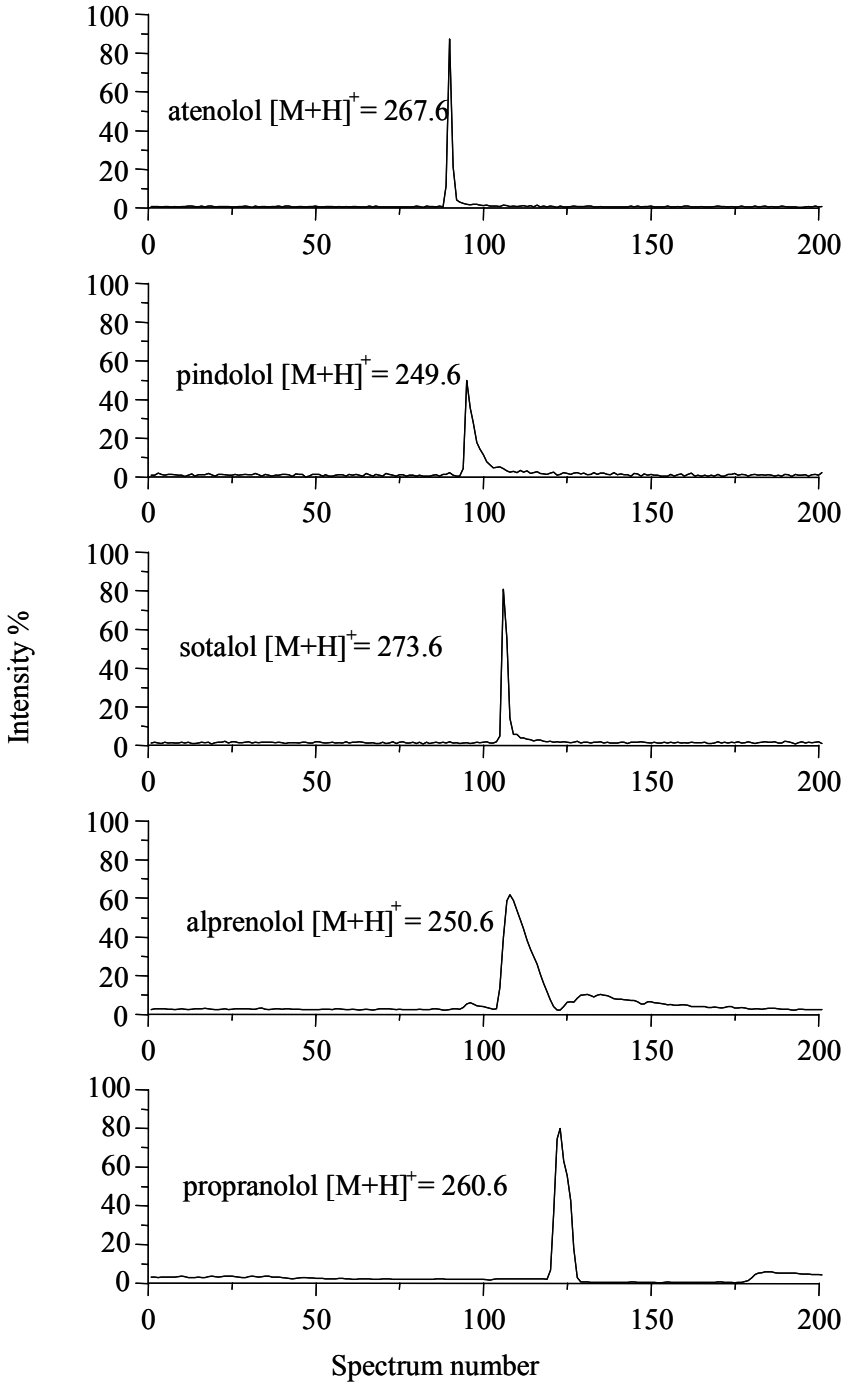

Figure 4.5 Selected ion chromatograms of five β-blockers obtained from OT-CEC/ESI MS. Conditions: PEM coating; 0.5% (w/v) PDADMAC dissolved in 0.2 M NaCl and 1% (w/v) poly-SUS; Buffer: 10 mM NH_4Ac (pH 9); CE separation voltage: 30 kV; capillary: 61.5 cm total length, 50 μm i.d.

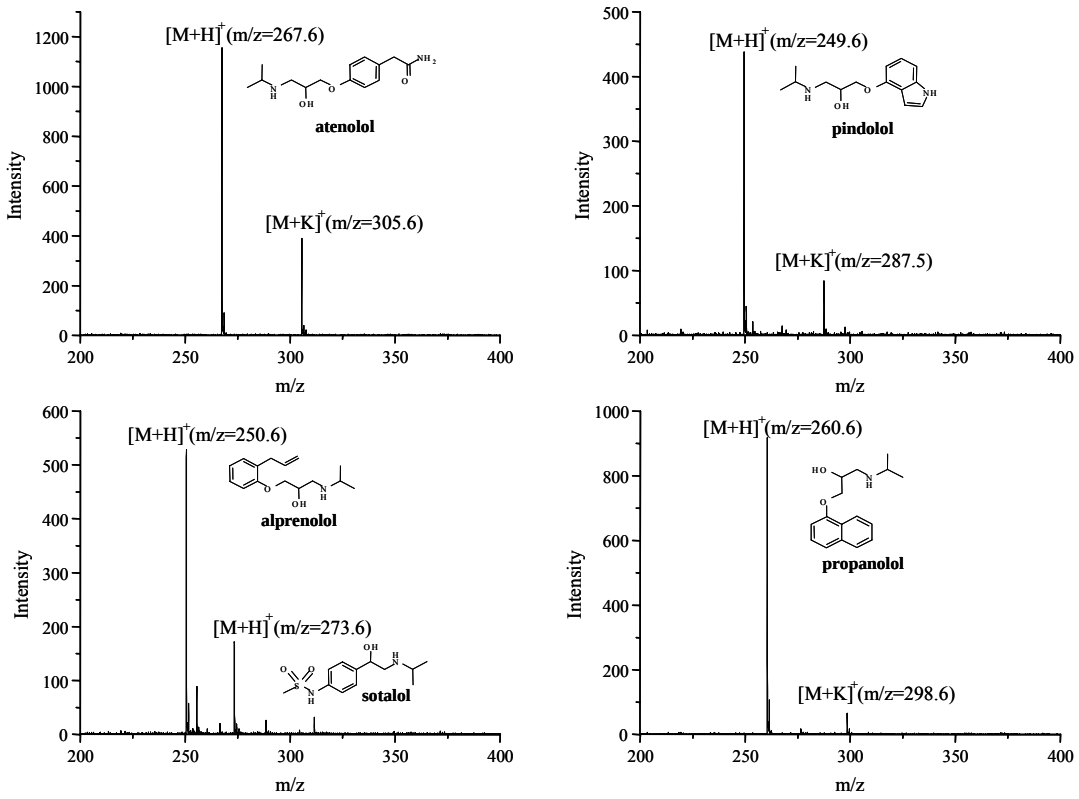

Figure 4.6 Mass spectra for the five β-blockers. The spectra were extracted individually from the selection ion chromatograms of the analytes shown in Figure 4.4a, a number of scans were averaged, and the background was subtracted.

4.4 Conclusion

The poly-SUS based PEM was used as a stationary phase in OT-CEC/ESI MS thereby preventing the surfactant from entering the mass spectrometer. MS contamination and surfactant mass signal interference problems inherent in MEKC/MS were eliminated and thus the method developed here was suitable alternative to MEKC/MS, giving rise to a favorable detection state for MS. Successful separation and detection of β-blockers and benzodiazepines was achieved.

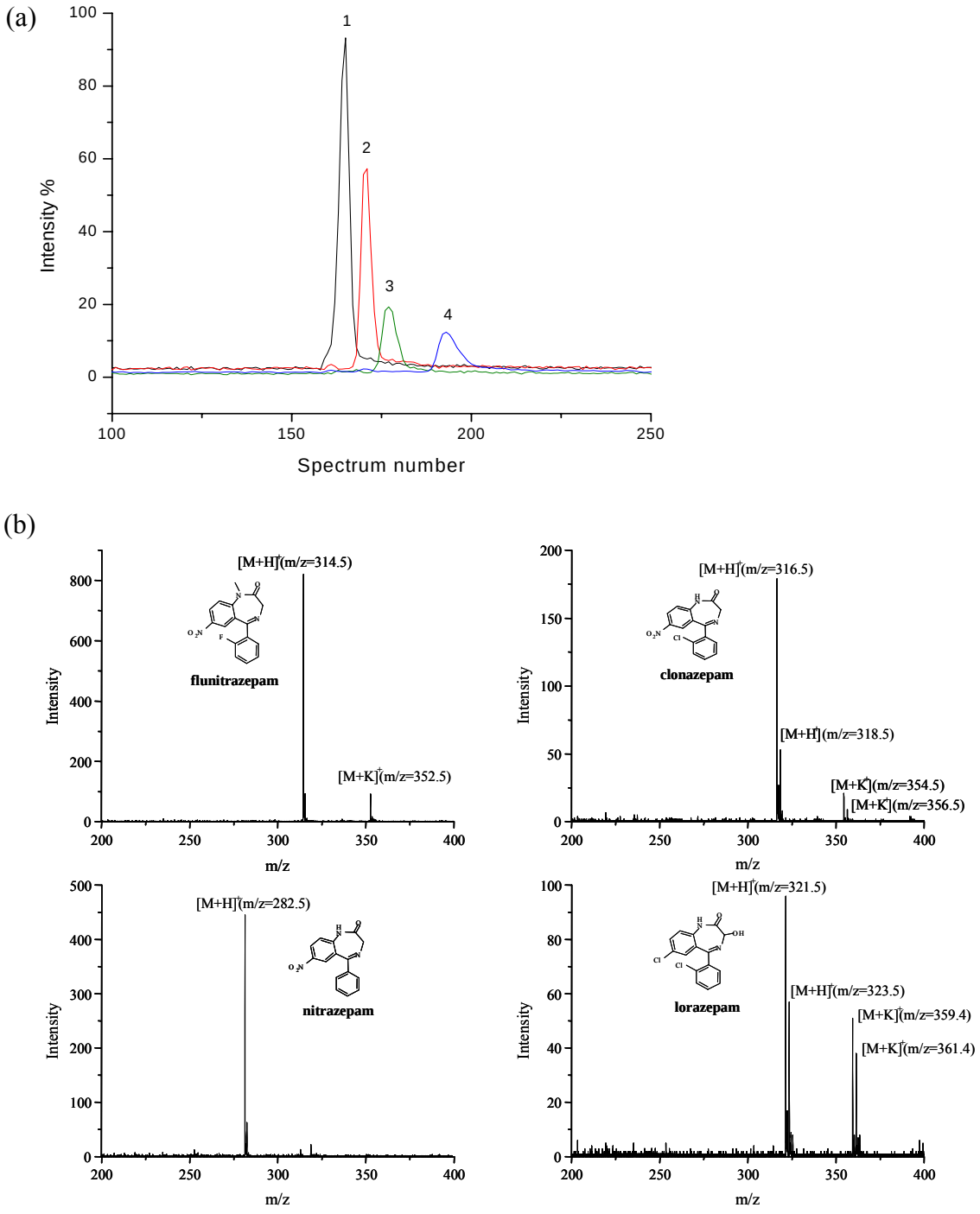

Figure 4.7 (a) Selected ion chromatograms of four benzodiazepines obtained from OT-CEC/ESI MS. CE separation voltage, 25 kV, buffer pH at 9.0. (b) Mass spectra for the four benzodiazepines. The spectra were extracted individually from the selected ion chromatograms of the analytes shown in (a), a number of a scans were averaged and the background was subtracted.

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CHAPTER 5

SIMULTANEOUS CONCENTRATION AND SEPARATION OF COUMARIN DYES USING A POLYMERIC SURFACTANT IN MICELLAR AFFINITY GRADIENT FOCUSING

5.1 Introduction

The use of equilibrium gradient focusing techniques, other than capillary isoelectric focusing (CIEF), is promising for analytical separations in the future and will provide viable alternatives to transient separation techniques such as capillary electrophoresis (CE) and chromatography [1-7]. This is because the former techniques can simultaneously separate and increase analyte concentration, thereby decreasing detection limits. Although CE as an analytical technique is popular because of its high peak efficiencies and low sample consumption, it suffers from poor detection limits when UV detection is used. Hence, equilibrium gradient focusing techniques provide promising alternatives to CE.

In equilibrium gradient focusing techniques, a force is induced by an external field, e.g. a magnetic or electric field, causing a gradient in the analyte's velocity along a separation channel [7, 8]. At a particular point along the separation channel, the net force can be adjusted such that the net mobility is zero and the analyte is focused at this point. The analyte preferentially migrates to this unique point and accumulates with time, resulting in concentration enhancement.

In contrast to stacking [9-11] and isotachophoresis [12, 13] concentration methods, where the velocity gradients are generated at buffer interfaces with varying ionic strengths, the point at which the focusing occurs in equilibrium gradient focusing techniques is stationary and does not move with the electroosmotic flow. Thus, equilibrium gradient focusing techniques are more amenable to miniaturization because the long separation channels required in

transient separations are not needed to achieve separation. In addition, equilibrium gradient focusing techniques do not exhibit analyte band broadening common with transient separations. Unlike transient techniques where the band width continually increases as analytes migrate along the separation column [1], the analyte separation bands become more narrow and concentrated with time in equilibrium gradient techniques.

Capillary isoelectric focusing (CIEF) [14-16] is the most commonly used equilibrium gradient technique. In this technique, analytes are focused at their respective isoelectric points (p*I*s) by the application of a pH gradient along the separation channel using various ampholytes. Although CIEF is a commonly used technique, its application is limited to analytes that have an accessible p*I* between pH 3 and 11. Thus, it is mainly used to separate proteins or peptides and has very limited use for separation of pharmaceutical compounds that exhibit high p*I* values. In this case, analytes must be chemically derivatized in order to achieve an accessible p*I*.

Electric field gradient focusing (EFGF) is a recently developed technique by Ivory and coworkers [3, 17-19] to overcome the some of limitations associated with the use of CIEF. In this technique, an electric field gradient is used to generate a velocity gradient across the column. The focusing of analytes is achieved by balancing the bulk flow rate with the electrophoretic velocity such that the net velocity is zero at a point where the analyte will be focused. Separation of analytes occurs as a result of differences in their electrophoretic mobilities. Although this technique can be applied to any analyte possessing an electrophoretic velocity (any charged analyte), the application of an electric field gradient is quite challenging since it involves the placement of a series of electrodes and a semipermeable membrane along the separation channel to create a non-uniform electric field.

Temperature gradient focusing (TGF) was recently developed by Ross and coworkers [5, 6, 20] and is in some ways simpler to implement than EFGF. This technique is similar to EFGF where analytes are focused by balancing the bulk flow against the electrophoretic velocity of an analyte; however, a temperature gradient is applied instead of an electric field gradient. The temperature gradient is created by heating one end of the separation channel and cooling the other. A suitable buffer with a temperature dependent ionic strength is used. TGF is limited in application to charged analytes and, thus, cannot be used for the separation of neutral compounds. In addition, in TGF the buffer selection is limited to those buffers that exhibit a temperature dependent ionic strength.

To overcome the limitations of TGF, Ross and coworkers introduced micellar affinity gradient focusing (MAGF) [4]. In MAGF, a pseudostationary phase is used to create a retention gradient based on a temperature dependent phase ratio and partition coefficient of the pseudostationary phase. On one end of the retention gradient, where the pseudostationary phase is at a high concentration, the analyte exhibits a high retention while at the other end of the retention gradient, there is low analyte retention. This technique is essentially a combination of micellar electrokinetic chromatography (MEKC) [21, 22] and TGF [5]. Similar to MEKC, a pseudostationary phase is added into the mobile phase and analytes are separated based on their interaction with the pseudostationary phase. The mode of operation of MAGF is similar to TGF with the main difference being that in MAGF the velocity gradient is created by the pseusdostationary phase. This contrasts with TGF where the velocity gradient is created by the temperature dependence of the ionic strength of the buffer solution. In both MAGF and TGF, the creation of respective gradients is applied by heating one end of the separation channel while cooling the other end.

The primary advantage of MAGF over TGF, EFGF, and CIEF is that the separation of both neutral and ionic compounds is possible. Unlike TGF, EFGF, and CIEF, the separation analytes in MAGF is based on the analyte properties other than the electrophoretic properties. Ross and coworkers [4] demonstrated the simultaneous separation and concentration of two rhodamine dyes using sodium dodecyl sulphate (SDS) micelles in MAGF. However, the main drawback in the use of conventional micelles such as SDS for MAGF is that the buffer has to be selected such that it favors micelle formation because of the dynamic equilibrium that exists between the monomer and the micelle. Thus, the use of polymeric surfactants in lieu of conventional micelles may be advantageous since they do not suffer from the dynamic equilibrium, mentioned above. In addition they can be used with any buffer and at lower concentrations than the critical micelle concentration (CMC).

Polymeric surfactants (molecular micelles) [23-26] have been used as suitable alternatives to conventional micelles as pseudostationary phases for MEKC separations due to their remarkable stability. The presence of covalent bonds, linking monomer molecules, eliminates the dynamic equilibrium that exists between monomer molecules and the normal micellar aggregate. Thus, unlike conventional micelles, polymeric surfactants do not have a CMC and can be used at very low concentrations that are below the CMC of conventional micelles. Furthermore, in contrast to conventional micelles where organic modifiers disrupt micelle formation, larger amount of organic modifier may be used without seriously affecting the properties of polymeric surfactants.

In this chapter, the use of the achiral polymeric surfactant, poly (sodium undecenyl sulfate), poly-SUS, in MAGF is investigated for the simultaneous focusing and separation of three coumarin dyes: coumarin 334 (C334), coumarin 450 (C450), and coumarin 460 (C460).

The coumarin dyes were chosen because they are neutral, hydrophobic, and fluorescent. These properties respectively allow us to examine nonionic species, high binding affinity to micelles, and excellent detection. The effect of varying the temperature gradient on the resolution of C334 and C460 is investigated in detail. In addition, the influence of a number of other parameters that affect separation and concentration enhancement are examined and discussed.

5.2 Experimental

5.2.1 Reagents and Chemicals

The coumarin laser dyes C460 and C450 were purchased from Exciton Inc (Dayton, OH) while C334 was purchased from Acros Organics (Geel, Belgium). The chemical structures of the analytes are shown in Figure 5.1.

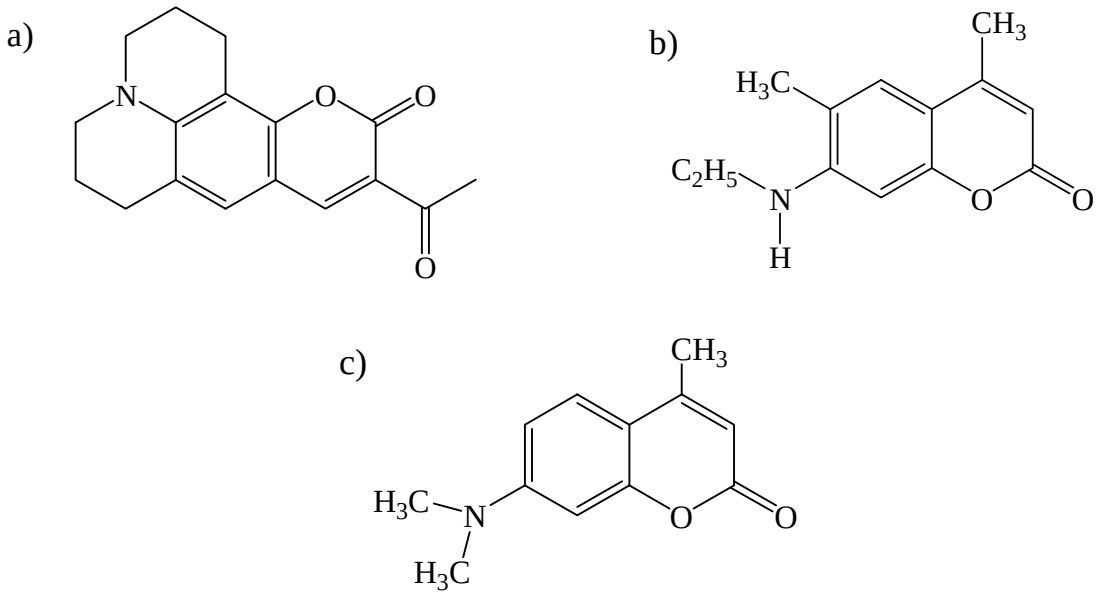

Figure 5.1 Structures of (a) C334, (b) C460, and (c) C450, coumarin dyes investigated.

All reagents were used as received and prepared in ultra-filtered water from Fisher Scientific (Fair Lawn, NJ). Sodium phosphate dibasic (Na_2HPO_4), sodium borate ($Na_2B_4O_7$), and sodium hydroxide (NaOH) were purchased from Sigma Chemical Company (St. Louis, MO).

Methanol (MeOH) was obtained from Aldrich (Milwaukee, WI). Poly-SUS was synthesized according to the previously reported procedure in Chapter 2, Section 2.2.2.

5.2.2 Buffer and Sample Preparation

The buffer solution consisted of 12.5 mM Na_2HPO_4, 12.5 mM $Na_2B_4O_7$, and 10% MeOH and was adjusted to 9.2 using 1 M NaOH. The appropriate amount of poly-SUS surfactant was added to the buffer. All solutions were filtered using 0.45 µm polypropylene nylon filters and sonicated before use. Stock solutions of the coumarin dyes were prepared by dissolving the dyes in pure methanol at a concentration of 500 µM. The final analyte concentration was prepared by dissolving with the appropriate amount of mobile phase.

5.2.3 Micellar Affinity Gradient Focusing Apparatus

5.2.3.1 Fluorescence Microscope

Fluorescence microscopy experiments were performed using a Leica DM LB fluorescence microscope equipped with a 10X objective lens and a mercury arc lamp. Microscope filter set used for detection of the coumarin dyes consisted of a 350± 25 nm band pass excitation filter and 420 nm long pass emission filter. All digital images were acquired using a color CCD camera (Dage-MTI22) using Scion Image software and a Scion CG-7 frame grabber (Scion, Inc., Frederick, MD).

5.2.3.2 Capillary Device Preparation

Separation of the coumarin dyes was performed using a capillary device (Figure 5.2) consisting of a 3-cm-long fused silica capillary (30 µm i.d., 360 µm o.d) purchased from Polymicro Technologies, LLC (Phoenix, AZ). A 5 mm optical window on the silica capillary was prepared by burning a portion of the outer polyimide coating. Thereafter, the capillary was embedded between two polycarbonate sheets obtained from McMaster Carr (Atlanta,

GA) by inserting the capillary, in a hydraulic press at 180 °C and 1000 lb for 5 min and cooling to 120 °C before releasing the pressure. To prevent crushing of the capillary and define the final thickness of the device, 2 metal shims were placed parallel to the capillary used in the press.

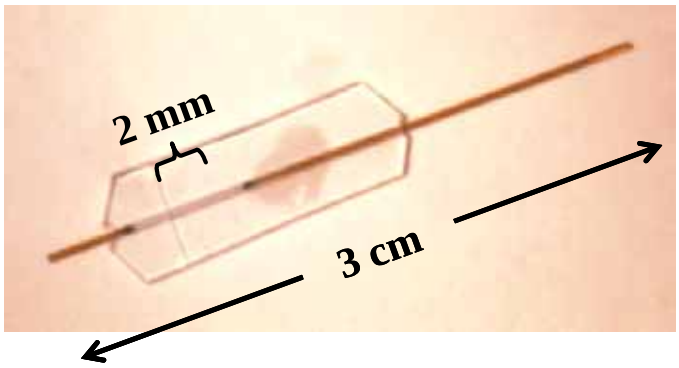

Figure 5.2 3cm long capillary device used for separation and focusing. The 2 mm region indicates the length of the gradient zone with which focusing and separation occurred.

During the focusing experiments, the 3 cm capillary device was mechanically anchored between two copper blocks. The higher temperature, T_H, of one end of the capillary device was regulated by use of a thermoelectric heated copper plate while the lower temperature, T_C, of the other end was regulated using a recirculation water bath. A schematic of the apparatus used in shown in Chapter, Figure 1.15. The length of the retention gradient zone was 2 mm as indicated in Figure 5.2. One end of the capillary was connected to a 150-μL volume sample reservoir while the other end was connected to the waste reservoir through a silicone rubber septum. The mobile phase was loaded into the microchannel through another reservoir attached by a nylon tube and anchored to a vertical translation stage. The hydrodynamic

pressure across the capillary device could be varied by adjusting the height of the translation stage.

Before each run, the microchannel was filled with the mobile phase and the sample reservoir with the analyte such that before application of the electric field, the microchannel and the reservoirs contained a sample or mobile phase solution of uniform concentration. A high voltage was then applied across the channel to simultaneously separate and concentrate the analytes.

5.3 Results and Discussion

5.3.1 Micellar Affinity Gradient Focusing

Separation and focusing of analytes occurs as a result of a retention gradient created by the interaction of the analyte with the polymeric surfactant and is explained by the retention

$$k = K\beta \tag{5.1}$$

where k is the retention factor of the polymeric surfactant, K is the partition coefficient that measures the affinity of the analyte to the polymeric surfactant and β is the phase ratio of the polymeric surfactant in the buffer solution. In this case, β is constant because the micelles used for these studies are polymerized and their volume relative to the mobile phase will remain constant with varying temperature. This is contrasts to the use of conventional micelles where the phase ratio changes with temperature.

5.3.2 Focusing and Separation of C334, C460, and C450

The primary advantage of MAGF is it ability to perform simultaneous concentration and separation. To illustrate this advantage, three neutral and hydrophobic coumarin dyes were focused and separated using poly-SUS (Figure 2.2, Chapter 2). Figure 5.3 shows the fluorescence micrographs obtained from the separation and focusing of a mixture of C334

(green), C460 (blue), and C450 (blue). The mobile phase used consisted of 0.125% w/v poly-SUS and was found optimal for these separations since higher concentrations of poly-SUS resulted in the analyte precipitating with the polymeric surfactant, which often led to capillary blockage. The separation resulted from differences in hydrophobic interactions with poly-SUS. Since the three coumarin dyes are neutral, such separation is also a result of hydrophobic interactions with poly-SUS.

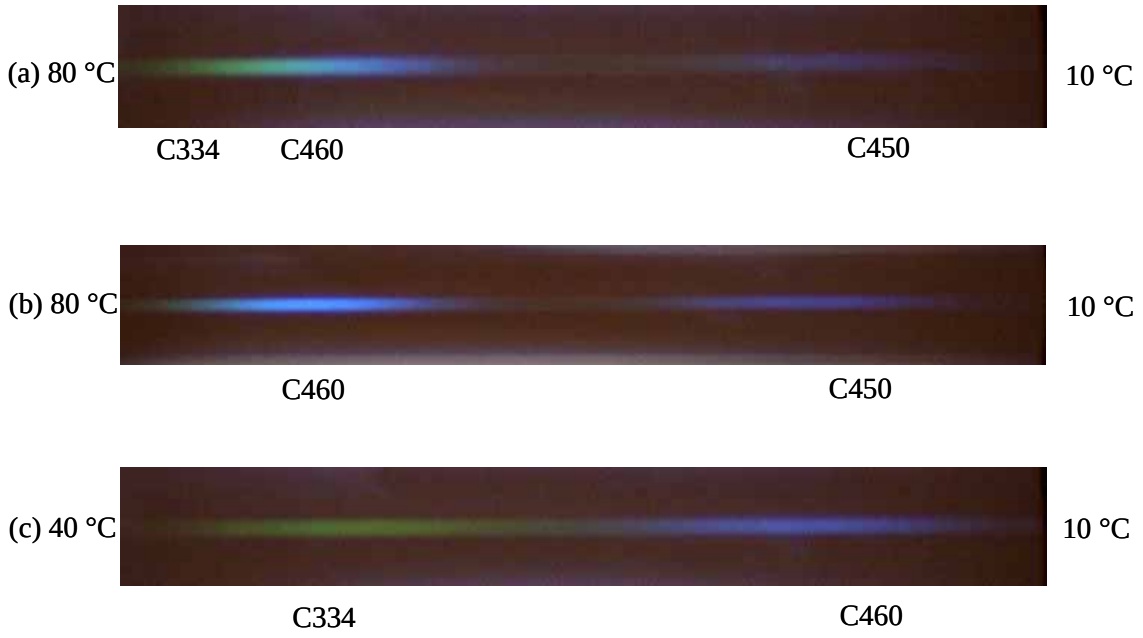

Figure 5.3 Fluorescence micrographs illustrating the focusing and separation of (a) C334 (green), C460 (blue), and C450 (blue), T_H = 80 °C and T_C = 10 °C (b) C460 and C450, T_H = 80 °C and T_C = 10 °C (c) C334 and C460, T_H = 40 °C and T_C = 10 °C. Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mM $N_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -2000V; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm. Initial analyte concentration, 25 nM

At a temperature gradient of T_H = 80 °C and T_C = 10 °C, C460 and C450 dyes were completely resolved; however, C334 and C460 overlapped at their focus points (Figure 5.3a). Figure 5.3b illustrates the separation and focusing of a mixture of C460 and C450. The C460

dye focuses at a faster rate than the C450 dye and thus appears brighter in this figure. At a lower temperature gradient of T_H = 40 °C and T_C =10 °C, C334 and C460 were completely resolved (Figure 5.3c).

5.3.3 Effect of Varying Temperature Gradient

The effect of a varying steepness (slope) of the temperature gradient on the resolution of the C334 and C460 was also investigated (Figure 5.4). In this study, the steepness of the temperature gradient was varied in steps by adjusting T_H of the microchannel from 80 °C to 30 °C while T_C was held constant at 10 °C and the retention gradient length was 2 mm. Once the desired temperature gradient was reached, a mixture of the two coumarin dyes was injected and a voltage was applied to achieve focusing.

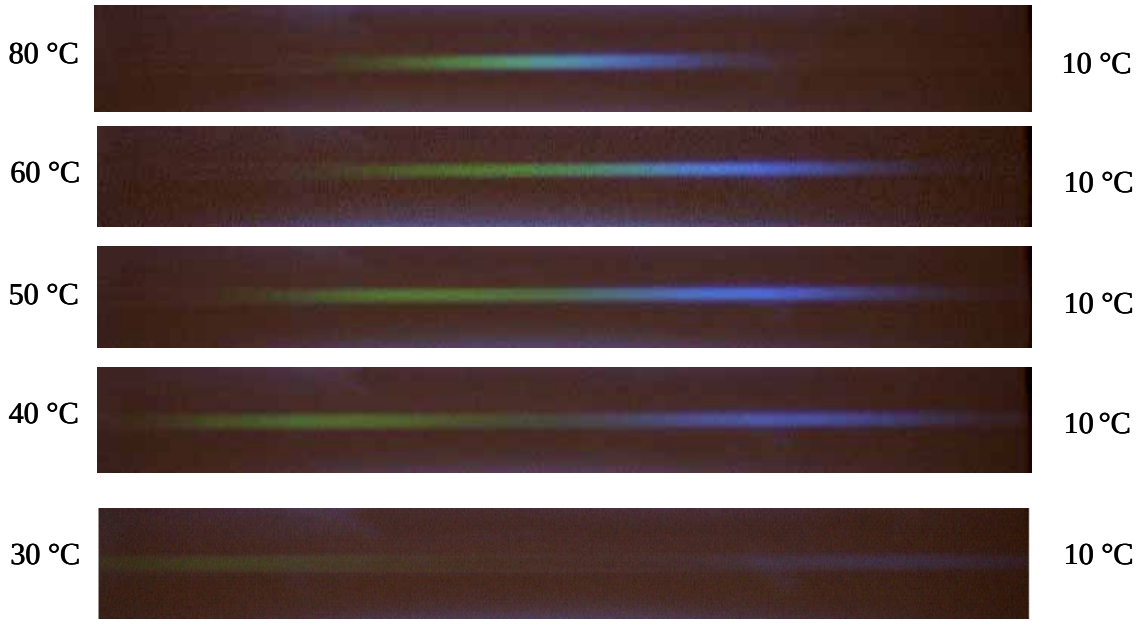

Figure 5.4 Fluorescence micrographs illustrating the effect of varying temperature gradient on resolution of C334 (green) and C460 (blue). Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -2000V; temperature gradient, T_H 80 °C, T_C 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm. Initial analyte concentration, 25 nM.

As shown in Figure 5.4, the resolution of the two coumarin analytes increased as the steepness of the temperature gradient was decreased. These static images were taken approximately 2 minutes after focusing. Since the interaction between a polymeric surfactant and an analyte is temperature dependent, the temperature gradient can be varied to enhance the resolution of analytes in MAGF. At a lower temperature gradient, better resolution is achieved with a slower rate of focusing. The use of a lower temperature gradient is equivalent to the use of longer capillaries in CE where better resolution is achieved at a longer migration time. Previous studies by Ross and coworkers [5, 6] indicate that higher temperature gradient results in a more rapid rate of focusing; however, low analyte resolution is observed.

5.3.4 Concentration Enhancement Studies

An advantage of MAGF is the ability to achieve concentration enhancement with an increase in focusing time. The concentration enhancement of C460 as a function of time was investigated, indicating a consistent gradual increase in the concentration with time (Figure 5.5). In this experiment, the initial analyte concentration was 5 nM and after focusing C460 for a time period of 10 minutes the final concentration was 1300 nM. Thus, under the experimental conditions used a concentration enhancement of 260-fold in 10 minutes was achieved. The concentration values in Figures 5.5 and 5.6 were determined from calibration plots prepared by running various standard concentrations of C460 and C334, respectively, along the capillary device under the same temperature and conditions (but with no voltage applied) and obtaining the average fluorescence intensities.

Figure 5.6 illustrates the fluorescence micrographs of the concentration enhancement of the C334 observed every two minutes during focusing. The initial concentration in this

experiment was 25 nM and a final concentration of 2500 nM resulting in a 100-fold enhancement in 10 minutes. It should be noted that further improvement in concentration enhancement can be achieved by varying separation parameters such as voltage, temperature gradient, and mobile phase composition. Preliminary results of the focusing of C334 using conventional micelles of SDS indicated a slower rate of focusing and broader peaks [27].

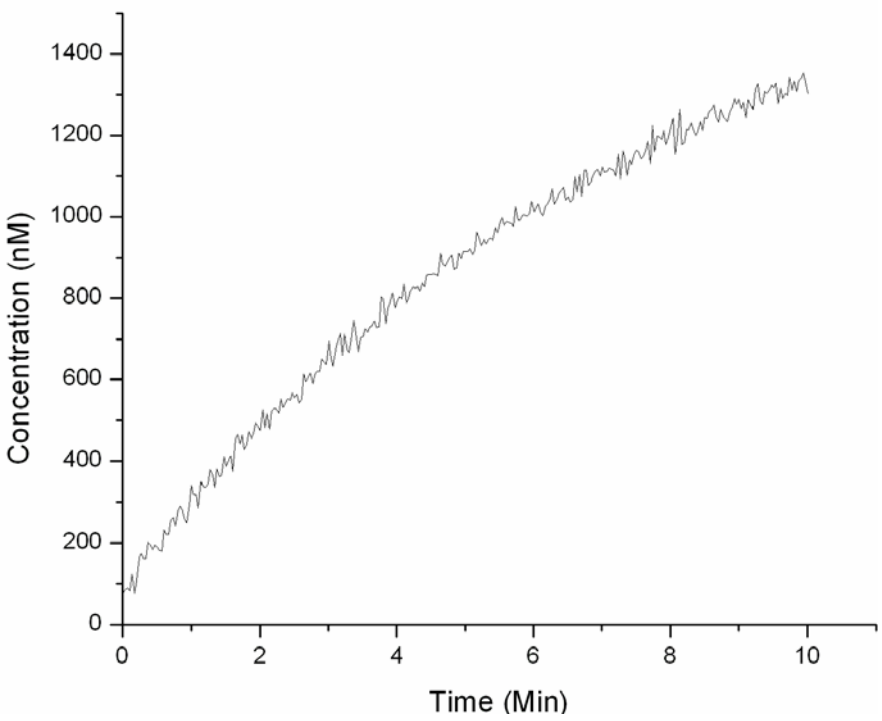

Figure 5.5 Plot of concentration as a function of time for C460. Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -1000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm. Initial analyte concentration, 5 nM; Analyte concentration after 10 minutes, 1300 nM.

5.3.5 Quantitative Focusing with Scanning MAGF

To evaluate the effect of input analyte concentration and focusing time on peak intensity and reproducibility, a series of scans were performed using a mixture of C460 and C450 analytes. Peak height and peak areas of the analytes were monitored as a function of time and

concentration. In order to enable the elution of the focused peaks of C460 and C450 past the detection point, the scans were performed by sequentially varying the bulk flow velocity of the buffer as a function of focusing (wait) time.

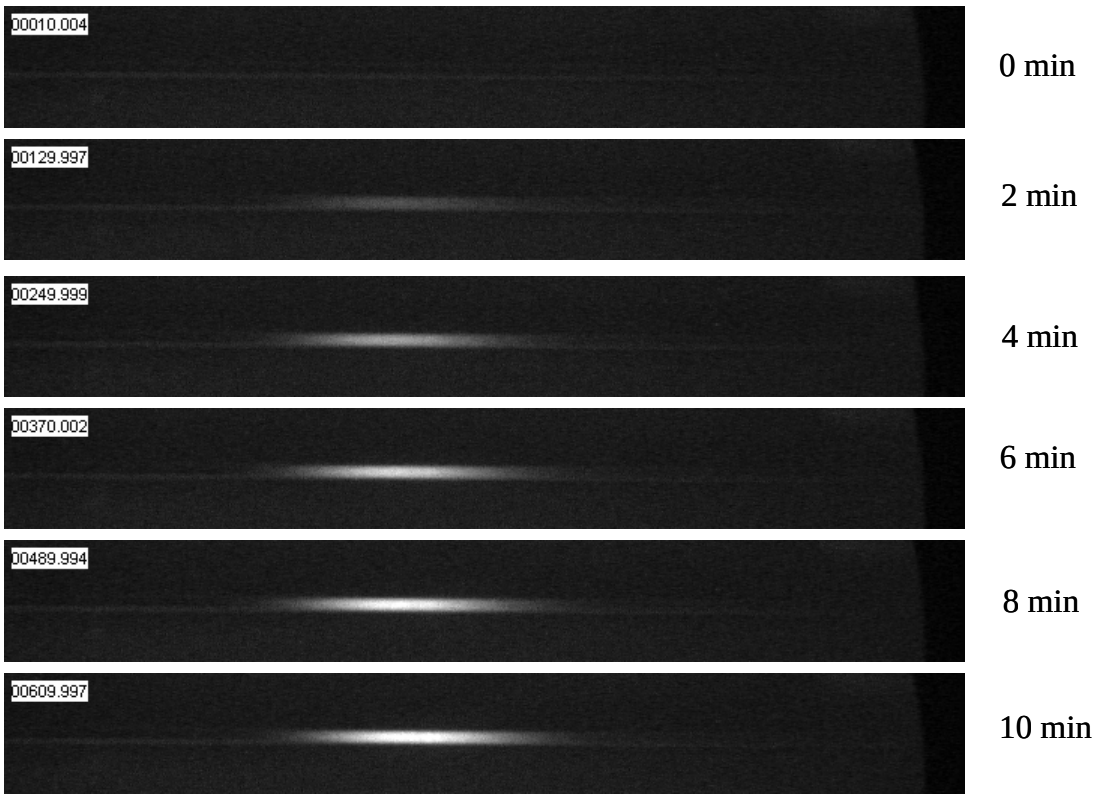

Figure 5.6 Fluorescence micrographs illustrating the focusing of C334 as a function of time. Images were taken in 2 minute intervals after voltage application. Focusing conditions: mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ and12.5 mM Na_2PO_4 at pH 9.2; voltage, -2000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm. Initial analyte concentration, 25 nM; Analyte concentration after 10 minutes, 2500 nM.

It should be noted that, because the bulk flow is a summation of the electroosmotic flow and hydrodynamic pressure exerted by the buffer [5], the bulk flow can be adjusted by varying the hydrodynamic pressure exerted on the buffer. In these experiments, the hydrodynamic pressure exerted by the buffer was adjusted by varying the height of the vertical translation stage by which the nylon tube containing the buffer was attached in steps of 3 mm. The

focusing time refers to the time interval allowed before a step of 3 mm was moved on the vertical translation stage. A plot of peak intensity versus the height of the buffer reservoir on the translation stage (the pressure applied to the waste end of the capillary) was used to represent the signal output recorded from these experiments. The peak intensity values were obtained from spot intensities of focused peaks using Image J software (National Institutes of Health, Bethesda, MD) and fitted on a Gaussian. Peak areas were obtained by integrating peak intensities while peak heights were measured at the maximum peak intensity.

The effect of varying input concentrations on the resulting peak intensities for a mixture of C460 and C450 was investigated and is illustrated in Figure 5.7a. As expected, an increase in analyte concentration resulted in an increase in peak intensities. The plots of peak area versus input concentration and peak height versus input concentration are shown in Figures 5.7b and 5.7c, respectively. Although the peak areas and peak height of the two coumarin dyes increased almost by the same magnitude for equivalent concentrations, a slight shift in peak position was observed (Figure 5.7a). Variation in hydrodynamic pressure exerted by the buffer and current may have resulted in a slight drift in peak positions.

A number of separation scans were performed to investigate the effect of varying focusing time on the peak intensities, areas, and heights of a mixture C460 and C450. As mentioned earlier, the focusing time in these experiments refers to the time interval allowed before a 3 mm step on the vertical translation was taken. Figure 5.8a is an illustration of peak intensities of a mixture of C460 and C450 at focusing time of 1.324 s, 2.324 s, and 3.324 s. The concentration of the mixture of C460 and C450 was 12.5 nM in this experiment. As expected, an increase in focusing time resulted in an increase in peak intensities. A plot of peak area and peak height versus focusing time is illustrated in Figures 5.8b and 5.8c,

respectively. From the figures, it is observed that the increased peak intensities were not parallel. In fact, the identity of the most intense peak changes from C460 to C450 at a focusing time between 2.324 s and 3.324 s. Thus, the rate of concentration enhancement of the two coumarin dyes is different; however, the reason for this observation is not clear.

The effect of varying focusing time at 1.324 s, 2.324 s, 5.324 s, and 10.324 s was investigated for the separation of C460 and C450 at a concentration of 5 nM each (Figure 5.9a). A plot of peak area and peak height versus focusing time illustrated in Figures 5.9b and 5.9c, respectively, indicated a slightly faster rate of focusing of C460 relative to C450 at a focus time of 1.324 s. For a focus time of 2.324 s C460 focused at the same rate as C450; however, with an increased focusing time from 5.324 s to 10.324 s, C450 focused faster than C460. Based on these observations, it may be deduced that the rate of focusing of the two analytes is dependent on the focusing time. With increasing focusing time, C450 focused faster than C460 at input analyte concentration of 5nM (Figures 5.9) and 12.5nM (Figure 5.8).

5.3.6 Reproducibility Study

The reproducibility of separations is a key factor for the evaluation of the performance of MAGF quantitative assays. Studies were preformed to assess the reproducibility of MAGF by calculating the relative standard deviation (RSD) of the peak height and peak area of a mixture of the C460 and C450. Figure 5.10 is an illustration of the run-to-run reproducibility of the separation and focusing of a mixture of C460 and C450 from three consecutive scans at a focusing time of 1.324 s. The RSD of the peak height and peak areas of C460 were found to be 6.19 % and 4.11 %, respectively, while those of C450 were 2.44 % and 6.82 %, respectively.

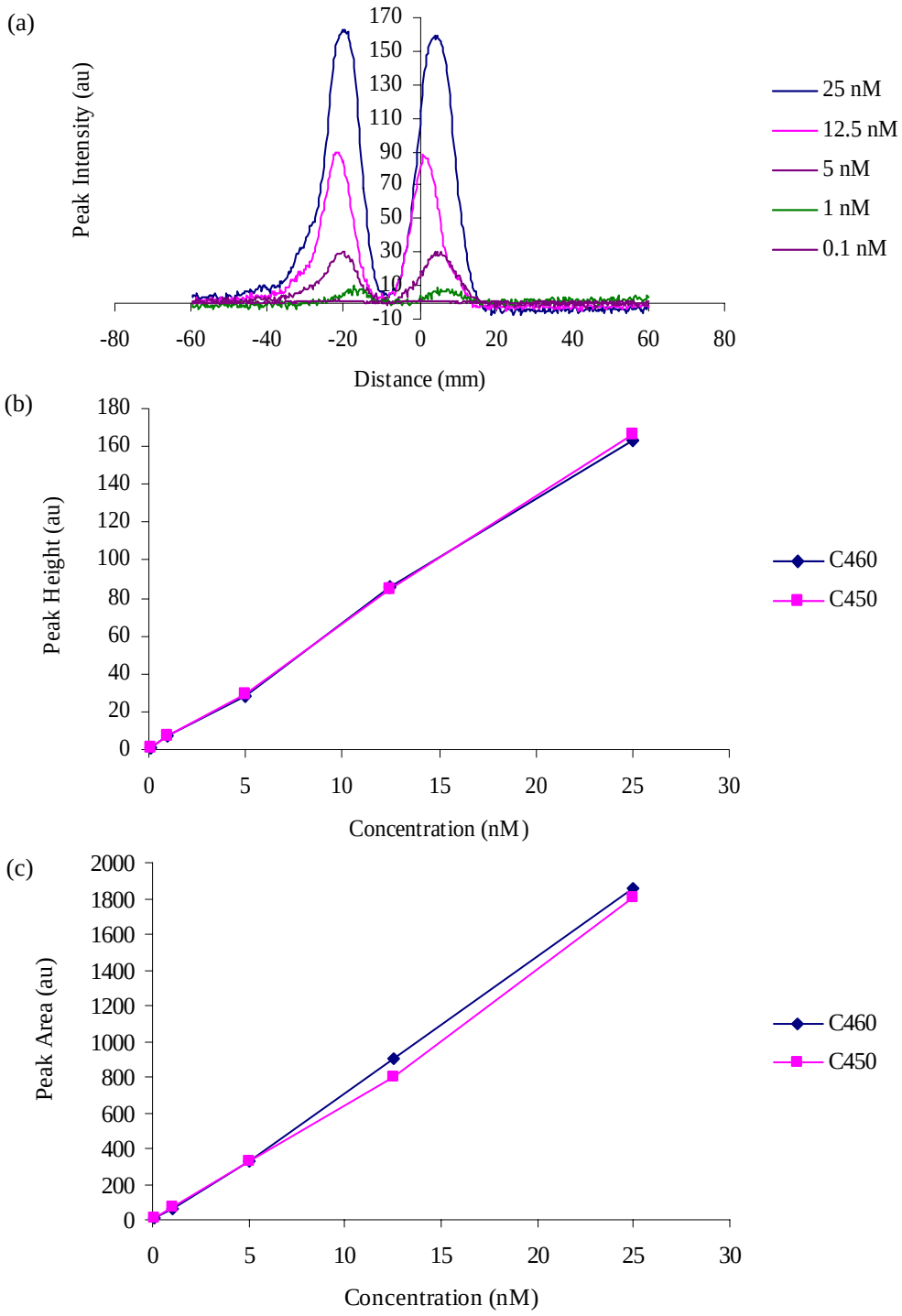

Figure 5.7 Effect of varying input concentration of C460 (left peak) and C450 (right peak) on (a) Peak Intensity (b) Peak Area (c) Peak Height. Focusing conditions: Focus time, 2.324s; mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -1000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm.

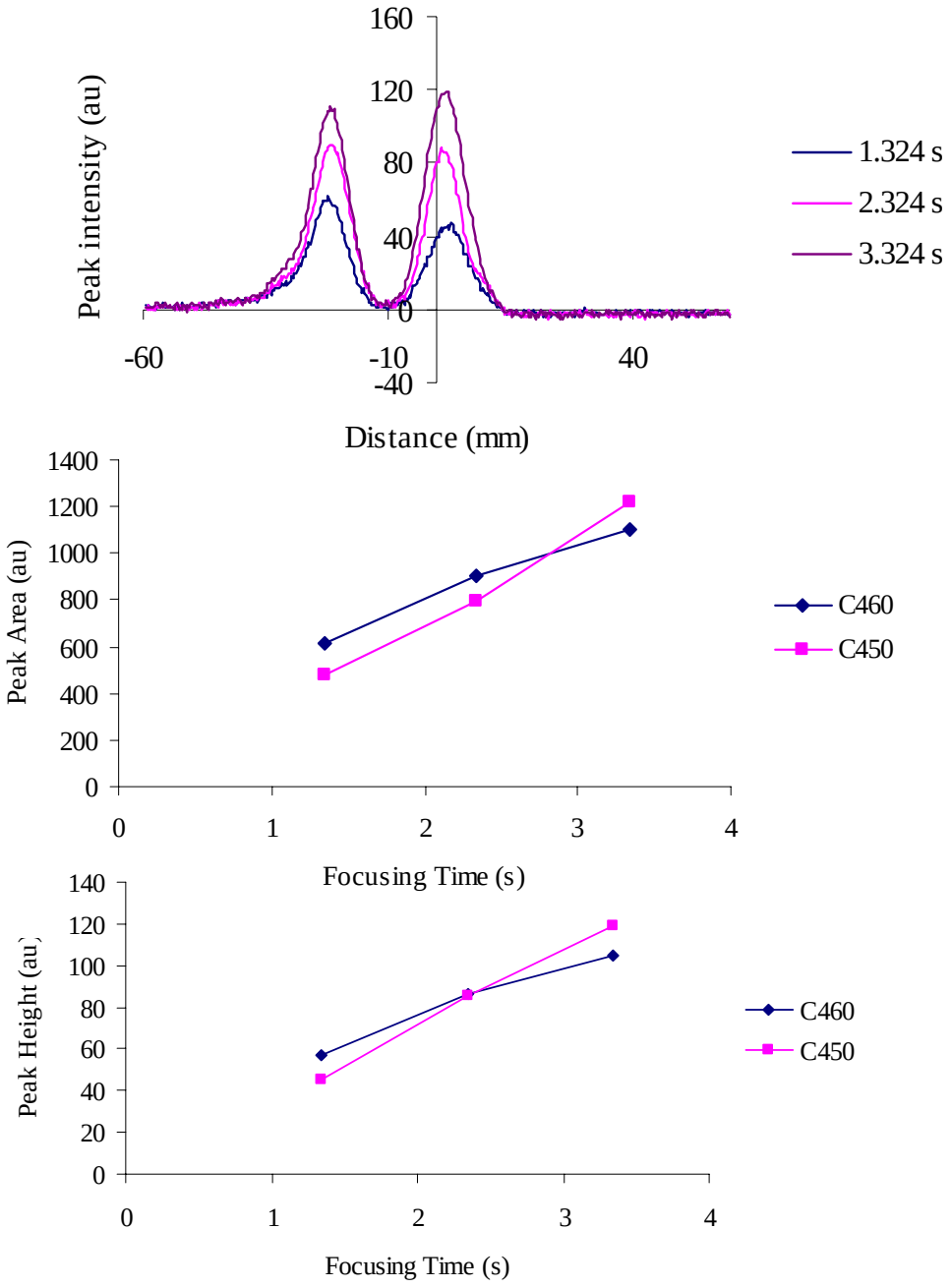

Figure 5.8 Effect of increasing focusing time of C460 (left peak) and C450 (right peak) each on (a) Peak Intensity (b) Peak Area (c) Peak Height at 12.5 nM concentration. Focusing conditions: focusing time, 1.324 s – 3.324 s; mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ 12.5 mM Na_2PO_4 at pH 9.2; voltage, -1000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm.

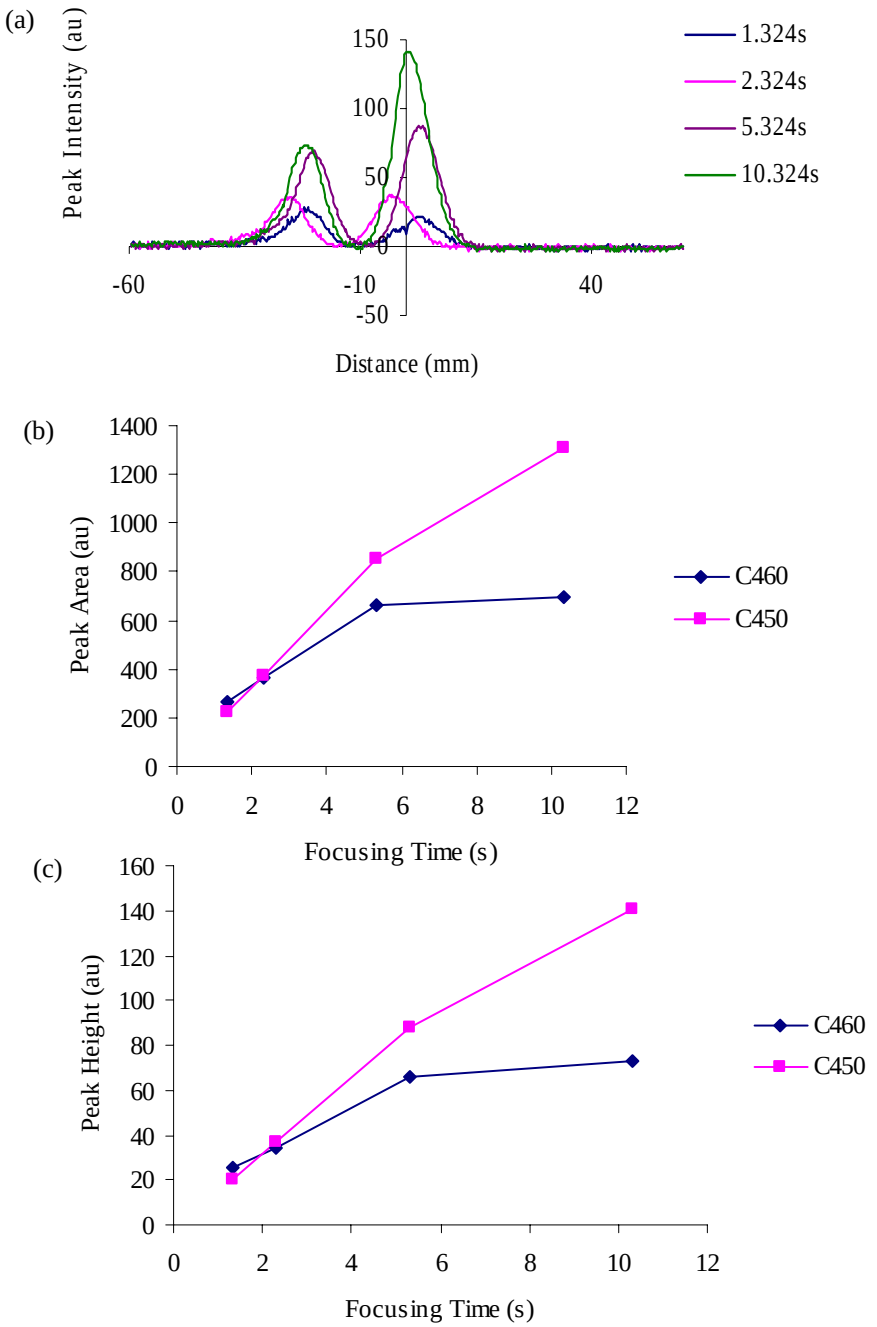

Figure 5.9 Effect of increasing focusing time of C460 (left peak) and C450 (right peak) each on (a) Peak Intensity (b) Peak Area (c) Peak Height at 5 nM concentration. Focusing conditions: focusing time, 1.324 s – 10.324 s; mobile phase, 0.125% w/v poly-SUS, 12.5 mM $Na_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -1000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d; gradient zone, 2 mm.

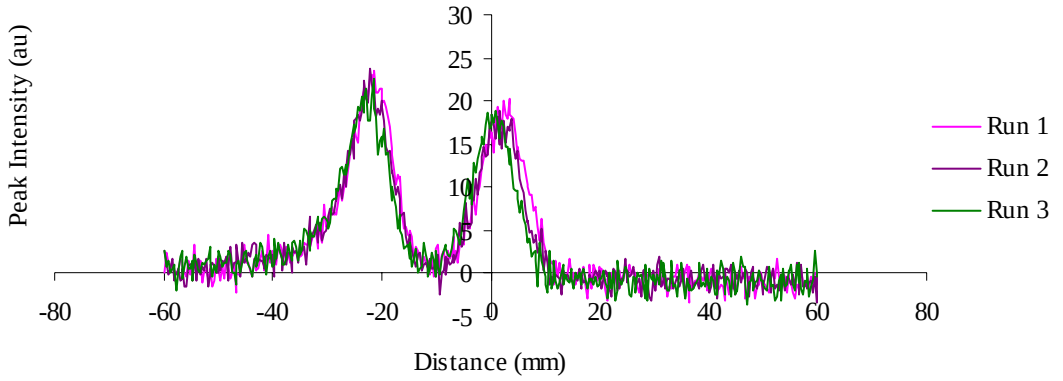

Figure 5.10 Run-to-run reproducibility of C460 (left peak) and C450 (right peak). Focusing conditions: focusing time, 1.324s; mobile phase, 0.125% w/v poly-SUS, 12.5 mM and $Na_2B_4O_7$ and 12.5 mM Na_2PO_4 at pH 9.2; voltage, -1000V; temperature gradient, T_H = 80 °C, T_C = 10 °C; capillary, 3 cm × 30 μm i.d, gradient zone, 2 mm. Analyte concentration, 5 nM each.

For a focusing time of 2.324 s, the RSD values of the peak height and peak areas of C460 were found to be 9.87 % and 9.67 %, respectively, while those of C450 were 5.07 % and 7.44 % respectively. The RSD values obtained in this study were unexpectedly high and were possibly due to error in repositioning the capillary device at the exact same position after each focusing and separation run and fluctuations in the brightness of mercury arc lamp used.

5.4 Conclusion

The simultaneous separation and concentration of three coumarin dyes using a polymeric surfactant has been demonstrated for the first time. Decreasing the steepness of the temperature gradient was shown to increase resolution between C334 and C460. Reproducibility of C460 and C450 peak heights and peak areas calculated by %RSD were found to be relatively high ranging from 2.44 to 9.87 %. The increase in peak intensity of the two coumarin dyes was dependent on the analyte concentration as well as the focusing time used.

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CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary

The research presented in this dissertation has focused on novel applications of polymeric surfactants in various analytical separation modes. These have included open-tubular capillary electrochromatography (OT-CEC), open-tubular capillary electrochromatography coupled to mass spectrometry (OT-CEC/MS), and micellar affinity gradient focusing (MAGF). The polymeric surfactants synthesized and investigated for these studies included both achiral and chiral based polymeric surfactants.

In Chapter 2, the use of an achiral polymeric surfactant, poly (sodium undecylenyl sulfate), poly-SUS, as a stationary phase coating in OT-CEC was examined. The experiments involved the *in situ* construction of an achiral polyelectrolyte multilayer (PEM) coating by the alternate adsorption of oppositely charged polyelectrolyte solutions on fused-silica capillaries. In this case, poly-SUS, was used as the negatively charged polyelectrolyte, while the commercially available polymer, poly (diallyldimethylammonium chloride), PDADMAC, was used as the positively charged polyelectrolyte. The performance of the PEM coating for separations in OT-CEC was evaluated by electrochromatographic experiments and showed good selectivity for both phenol and benzodiazepine analytes. The significance of the PEM coating was illustrated by comparing separations obtained on an uncoated fused-silica capillary and a PEM coated capillary. In the uncoated fused-silica capillary, no separation was obtained for benzodiazepines while in the separation of phenols shorter elution times and resolution of six out of the seven analytes were observed. Separations of the benzodiazepine analytes using the PEM coating were also compared to

those in MEKC. Although peak efficiencies obtained in MEKC were better than with PEM coating, longer elution times were observed and two of the analytes could not be resolved. Finally, the reproducibility of the coating was evaluated by calculating the relative standard deviation (RSD) of the electroosmotic flow (EOF). The run-to-run and capillary-to-capillary RSD values of the EOF were found to be less than 1.5%.

In Chapter 3, a chiral PEM coating consisting of the polypeptide, poly-L-lysine hydrobromide, poly-L-lysine, and the polymeric dipeptide surfactant, poly (sodium undecanoyl-L-leucine alanate), poly-L-SULA, was investigated as a new medium for the separation of chiral analytes in OT-CEC. In this approach, a stable PEM is constructed *in situ* by alternate rinses of the cationic polymer and the anionic polymeric surfactant. In previous studies, the PEM coating has been constructed by use of the cationic achiral polyelectrolyte, PDADMAC. In this study, the use of a chiral biopolymer, poly-L-lysine, as the cationic polyelectrolyte in the PEM coating was investigated. The results obtained indicated an increase in selectivity and resolution when poly-L-lysine is used as the cationic polymer in place of PDADMAC. In order to evaluate the chromatographic performance of the PEM coating as a chiral stationary phase, the separation of the β-blockers labetalol and sotalol, and the binaphthyl derivatives 1,1′-bi-2-naphthyl-2,2′-dihydrogen phosphate (BNP), 1,1′-bi-2-naphthol (BOH) and 1,1-binapthyl-2,2′-diamine (BNA) was investigated. In addition, the effect of varying the amino acid order of the polymeric dipeptide surfactant on resolution was examined and found to have an effect on the selectivity of the PEM coating. The number of bilayers also significantly influenced the separation efficiency and resolution of enantiomers. The run-to-run and capillary-to-capillary reproducibilities were evaluated by calculating the RSD of the EOF and were found to be less than 1%. The coating was stable and allowed

more than 290 runs to be performed in the same capillary. In addition, coupling of this chiral OT-CEC column with MS was investigated and shown to be successful.

In Chapter 4, the use of the PEM coating detailed in Chapter 2 was examined as a stationary phase coating enabling the coupling OT-CEC with ESI MS . The motivation of coupling the PEM to ESI MS was to develop a method aimed at minimizing the introduction of the pseudostationary phase or polymeric surfactant into the MS that occurs when MEKC is coupled to ESI MS. In so doing, the detection interference caused by the nonvolatile polymeric surfactant in MS is eliminated. In this work, the separation and detection of β-blocker and benzodiazepine analytes was investigated. The effect of buffer pH and applied voltage on the separation of these analytes was explored and under optimal conditions four of the five β-blockers and four benzodiazepines were separated and detected using this technique.

In Chapter 5, the use of poly-SUS for the simultaneous separation and concentration of hydrophobic and neutral compounds using MAGF. The use of MAGF was advantageous as it combined favorable features of both micellar electrokinetic chromatography (MEKC) and temperature gradient focusing (TGF) to achieve separation and focusing of fluorescent analytes. In this study, three coumarin dyes, coumarin 334 (C334), coumarin 450 (C450), and coumarin 460 (C460), were simultaneously separated and concentrated using poly-SUS as the pseudostationary phase. The effect of varying the steepness of the temperature gradient in the separation of C334 and C460 was investigated. Preliminary data comparing of focusing of C334 by use of the conventional surfactant sodium dodecyl sulfate (SDS) with poly-SUS indicates slower focusing and broader peak when SDS micelles were used. The effect on input concentration and focusing time on peak intensity was also studied. To evaluate the

reproducibility of the injections, the RSD of peak position, peak height, and peak area was computed and found to be less than 10%.

6.2 Future Directions

Studies concerning the combined use of OT-CEC [1-3] and MEKC [4, 5] should be investigated in the future. The motivation to investigate this work originates from previous studies comparing separations performed using the MEKC mode of separation to those performed in OT-CEC using PEM coated capillaries [6]. While shorter elution times were observed for separations performed on PEM coatings, lower peak efficiencies were also obtained in analytes containing polar groups resulting from analyte interactions with the coating [6-9]. Separations performed using MEKC yield high peak efficiency separations at shorter elution times. Thus, by combining MEKC and OT-CEC we should minimize the elution times and improve peak efficiencies.

Preliminary studies investigating the separation of benzodiazepines using combined MEKC and OT-CEC are illustrated in Figure 6.1. In this study, a fused-silica capillary coated with 1 bilayer of PDADMAC and poly-SUS using the coating procedure detailed in Chapter 2, Section 2.2.5. Thereafter, separations of benzodiazepine analytes (Figure 2.4, Chapter 2) were performed using a mobile phase containing varying concentrations of poly-SUS. By increasing the concentration of poly-SUS in the mobile phase an increase in resolution as well as elution time is observed. A comparison of the separations obtained in Figure 6.1 and OT-CEC (Figure 2.10b, Chapter 2) separations in indicates an improvement in peak efficiencies.

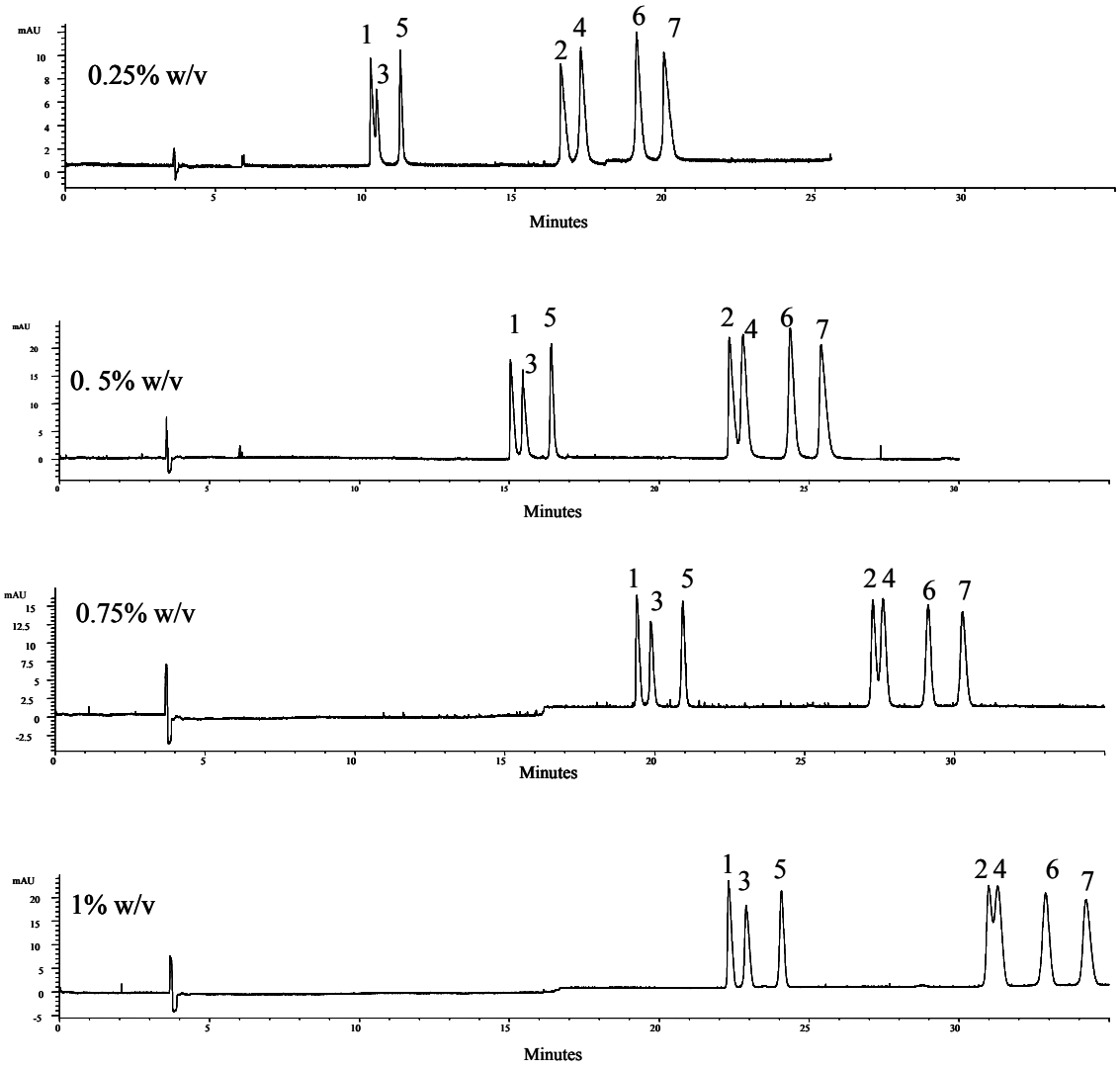

Figure 6.1 Effect of poly-SUS concentration in mobile phase in combined OT-CEC/MEKC, 1 Bilayer coating: PDADMAC 0.5% (w/v), poly-SUS (0.25-1) % (w/v), Conditions: Mobile phase: 25 mM $Na_2HPO_4/Na_2B_4O_7$ (pH 9.2); temperature; 20 °C; voltage, 20 kV; capillary: 58 cm total length, 50 cm effective length, 50 μm i.d.; detection 254 nm. Analyte peaks are labeled according to the elution order as indicated in Figure 2.4, Chapter 2.

Further studies investigating the use of chiral polymeric surfactants in MAGF for the separation and focusing of chiral analytes will be investigated. The chiral polymeric surfactant poly-SULV [10] has been shown to a versatile chiral selector in MEKC

separations and thus, it is anticipated that MAGF separations performed using poly-SULV will provide good selectivity and resolution.

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APPENDIX

LETTERS OF PERMISSION

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VITA

Mary Waithira Kamande was born in Nairobi, Kenya, on May 8, 1976, to Winny Wamaitha and Peter Nga'nga Kamande. She is the third born child in a family of four children, with an elder brother and sister, and a younger sister. She attended primary school at Westlands Primary School and thereafter was admitted to a private Catholic high school, Kianda School. After obtaining her high school diploma in 1995, she attended an international baccalaureate program for one year at the St. Mary's School in Nairobi. In March 1996, Mary was admitted to the Jomo Kenyatta University of Agriculture and Technology (JKUAT) for her undergraduate studies. She obtained a Bachelor of Science degree in chemistry with second class honor (upper division) in March 2000. During the course of her undergraduate studies, she interned with East Africa Industries, a Unilever company in Kenya. In addition to her curriculum at JKUAT, she performed independent research under the guidance of Professor Antony Gachanja on the determination of trace metals and free fatty acids in commercially available vegetable fats and oils.

In the fall of 2000, Mary was admitted to Louisiana State University to pursue her doctoral studies in the chemistry department under the guidance of Professor Isiah Warner. In her final year of graduate studies, she was awarded a Pfizer Research Fellowship based on her excellence in research and coursework. She was also the recipient of the James Robinson Award for excellence in analytical chemistry research. In addition, she spent three months at the National Institute of Standards and Technology in Gaithersburg, Maryland, working on a collaborative research project with Dr. Laurie Loscascio and Dr. David Ross. Her dissertation focuses on novel applications of polymeric surfactants for analytical separations.

The following is a list of excerpts from her dissertation research work that have been published and a patent she co-invented during her graduate studies.

- **Kamande, M. W.**, Ross, D., Loscasio, L. and Warner, I.M. "Micellar Affinity Gradient Focusing Using Polymeric Surfactants" in preparation for publication.

- **Kamande, M. W.**, Fletcher, K. A., Lowry, M and Warner I. M. "Analytical Separations Using Polyelectrolyte Multilayer Coatings in Capillary Electrochromatography" *J. Sep. Sci*, 2005, 28, 710-718.

- **Kamande, M. W**.; Zhu, X.; Kapnissi-Christodoulou, C. P. and Warner I. M. "Chiral Separations Using a Polypeptide and Polymeric Dipeptide Surfactant Coating in Open-Tubular Capillary Electrochromatography" *Anal. Chem.* 2004, 76, 6681-6692.

- **Kamande, M. W**., Kapnissi, C.P, Akbay, C. and I. M. Warner "Open-tubular Capillary Electrochromatography Using a Polymeric Surfactant Coating" *Electrophoresis*, 2003, 24, 945-951.

- Zhu, X.; **Kamande, M. W**.; Thiam, S.; Kapnissi, C.P.; Mwongela, S. M. and Warner, I. M. "Open-Tubular Capillary Electrochromatography/Electrospray Ionization Mass Spectrometry Using a Polymeric Surfactant as a Stationary Phase Coating" *Electrophoresis*, 2004, 25, 562-568.

Patent:

- Warner, I. M.; Kapnissi, C. P.; **Kamande, M. W**.; Valle, B.C.; Analytical Separations with Polyelectrolyte Layers, Molecular Micelles, or Zwitterionic Polymers. Patent No. 10/283,47

During her graduate studies she presented her research work at a number of national scientific conferences including (presenting author is underlined):

- "Improved Separations in Micellar Electrokinetic Chromatography Using Polyelectrolyte Multilayer Coated Capillaries and Polymeric Surfactants" **Mary W. Kamande**, Simon M. Mwongela, Linda S. Wabuyele, and Isiah M. Warner. 229th ACS National Meeting, San Diego, CA, March 2005

- "Analytical Separations Using Polymer-Coated Capillaries and Polymeric Surfactants in Micellar Electrokinetic Chromotography." **Mary W. Kamande**, Simon M. Mwongela, Linda S. Wabuyele, and Isiah M. Warner. Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), Orlando, FL, March 2005

- "Separation of Prostaglandin E2 and E3 in MEKC Using a Polymeric Surfactant as a Pseudostationary Phase." Xiaodong Huang, Xiaofeng Zhu, **Mary W. Kamande**, Yvonne M. Denkins, Isiah M. Warner. Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), Orlando, FL, March 2005

- "Enantiomeric Separation using Polymeric Surfactants in Open-Tubular Capillary Electrochromatography**.**' Tarus J, Kapnissi C.P., **Kamande M.W**., Warner I.M. 227th ACS National Meeting, Anaheim, CA, March, 2004

- "Chiral Separation of Binaphthyl Derivatives and Betablockers Using Polyelectrolyte Multilayer Coated Capillaries." **Kamande, Mary W**, Zhu, Xiaofeng, Kapnissi-Christodoulou, Constantina P, and Warner, Isiah M. 31st Annual Conference of the National Organization for the Professional Advancement of Black Chemists and Chemical Engineers (NOBCChE), San Diego, CA, April 2004.

- "Chiral Separations Using a Polymeric Dipeptide Surfactant in Open-Tubular Capillary Electrochromatography" **Kamande, Mary W**, Kapnissi-Christodoulou, Constantina P, Zhu, Xiaofeng and Warner, Isiah M. Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), Chicago, IL, March 2004

- "Separation of Phenols and Benzodiazepines Using Poly (Sodium Undecylenic Sulfate) in Open-Tubular Capillary Electrochromatography" **Mary W. Kamande**, Constantina P. Kapnissi, Xiaofeng Zhu, Cevdet Akbay, and Isiah M Warner. 225th ACS National Meeting, New Orleans, LA, April 2003

- "Open-tubular Capillary Electrochromatography Using a Polymeric Surfactant Coating." **Mary W. Kamande**, Constantina P. Kapnissi, Xiaofeng Zhu, Cevdet Akbay, and Isiah M Warner. Pittsburg Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon), New Orleans, LA, March 2003

- "Open-Tubular Capillary Electrochromatography Using a Polymeric Surfactant Coating." **Mary W. Kamande**, Constantina P. Kapnissi, Xiaofeng Zhu, Cevdet Akbay, and Isiah M Warner. HPCE 2003 16th International Symposium on Microscale Separations and Analysis, San Diego, CA, January 2003.